To my loving family

Believe you can and you're halfway there.

(Theodore Roosevelt)

DANK JE WEL..

Nu ik aanbeland ben bij het laatste te schrijven deel van mijn thesis, overvalt me een dubbel gevoel. Toch is het vooral dankbaarheid dat op dit moment primeert, want ik was omringd door vele uitzonderlijke mensen, die elk op hun manier hebben bijgedragen om deze thesis tot stand te brengen.

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Ik hou niet van afscheid nemen maar ik zie dit doctoraat ook niet als een einde, maar eerder als een begin van een nieuwe mooie en verrassende reis

..wordt vervolgd..

Veel liefs,
Eva

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ACAT	Acyl co-enzyme A:cholesterol acyltransferase
Acetyl CoA	Acetyl coenzyme A
Allo-THF	5α-tetrahydrocortisol
AKR1D1	Aldo-keto-reductase member D1
APACHE	Acute physiology and chronic health evaluation
ApEn	Approximate entropy
AVP	Arginine vasopressine
BMI	Body mass index
cAMP	Cyclic AMP
CBG	Cortisol binding globulin
CE	Cholesterol esters
CIRCI	Critical illness related adrenal insufficiency
CLIP	Corticotropin-like intermediate peptide
CNS	Central nervous system
CK	Cytokeratin
CO	Cardiac output
CRE	cAMP response element
CREB	cAMP response element binding protein
CRH	Corticotropin releasing hormone
CRP	C-reactive protein
CYP11A1	Cytochrome P450 family11, SubfamilyA, polypeptide1
CYP11B	11β-Hydroxylase
CYP17	17 α-Hydroxylase
CYP21A2	21-Hydroxylase
cDNA	complementary DNA
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DNR	Do not resuscitate
E	Cortisone
EC_{50}	Effective concentration driving half-maximal secretion
EDTA	Ethylenediaminetetraacetic acid.

ERK	Extracellular signal-regulated kinase
F	Cortisol
FXR	Farnesoid X –receptor
GAPDH	Glyceraldehyde 3-phosphate dehydogenase
GC-MS	Gas-chromatography-mass-spectrometry
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HDL	High density lipoprotein
HE	Hematoxylin-eosin
HMGCR	3-hydroxy-3-methylglutaryl-co enzyme A reductase
HPA	Hypothalamic-pituitary-adrenal
HSD	Hydroxysteroid dehydrogenase
HSL	Hormone sensitive lipase
ICU	Intensive Care Unit
IMM	Inner mitochondrial membrane
IL	Interleukin
IQR	Interquartile Range
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LIF	Leukemia inhibitory factor
MC2R	Melanocortin type 2 receptor
MSH	Melanocyte stimulating hormone
MR	Mineralocorticoid receptor
MRAP	MC2R accessory protein
mRNA	messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NEFL	Neurofilament protein light polypeptide
ORO	Oil Red O
PBR	Peripheral type benzodiazepine receptor
PKA	Protein Kinase A
POMC	Pro-opiomelanocortin
PVH	Paraventricular hypothalamus
RAI	Relative adrenal insufficiency
RNA18S5	18S ribosomal 5 RNA

SCN	Supra-chiasmatic nucleus
SD	Standard deviation
SF-1	Steroidogenic factor 1
SIRS	Systemic inflammatory response syndrome
SCARB1	Scavenger receptor class B member 1
SRD5A	Steroid α-reductase family
STAR	Steroidogenic acute regulatory protein
THE	Tetrahydrocortisone
THF	5β-tetrahydrocortisol
TNF-α	Tumor necrosis factor alpha
V1b-receptor	Vasopressine 1b-receptor

CHAPTER 1

INTRODUCTION

Adapted from:

- Boonen E, Van den Berghe G. Endocrine Responses to Critical Illness: Novel Insights and Therapeutic Implications. J Clin Endocrinol Metab 2014 (in press)

- Boonen E, Van den Berghe G. Cortisol Metabolism in Critical Illness: Implications for Clinical Care. Curr Opin Endocrinol Diabetes 2014 (in press)

1.1 HYPOTHALAMIC PITUITARY ADRENAL AXIS

1.1.1 Normal HPA-axis regulation

Traditional concept of the stress response

All living species aim to maintain a complex dynamic equilibrium or homeostasis, which is constantly challenged by internal or external adverse stimuli, called stressors.[1, 2] Stress in physiological terms is therefore a state in which homeostasis is threatened and which should be restored by coordinated physiological processes, together called the stress response. This response is essential for survival. The stress response is exerted by the combination of the neuronal pathways, linked to the release of catecholamines from the adrenal medulla and the hypothalamic pituitary adrenal (HPA)-axis control to release glucocorticoid production by the adrenal cortex. These immediate stress responses comprise many orchestrated endocrine adaptations that are presumably directed towards providing the required energy for the 'fight or flight response' in a context of exogenous substrate deprivation. Indeed, alterations within the different hypothalamic pituitary axes bring about lipolysis, proteolysis and gluconeogenesis and redirect energy consumption towards those processes that mediate acute survival, while anabolism is postponed to more prosperous times.

To pursue this response a complex coordination of central and peripheral systems is indispensible. Whenever stress occurs different afferent signals activate the central nervous system (CNS). Consequently the CNS causes activation of the hypothalamus which initiates the release of the corticotropin-releasing hormone (CRH) and arginine vasopressine (AVP) which stimulates the anterior pituitary corticotrophs to secrete the adrenocorticotropic hormone (ACTH). ACTH induces the production and release of cortisol from the adrenal gland. Cortisol itself can turnoff this activation of the HPA-axis by negative feedback inhibition as such preventing the deleterious effects of excessive HPA-axis activation. (Figure 1)

Regulation of the hypothalamus

The hypothalamus integrates all stress signals and converts these neurologic signals into hormone responses. Different types of stress signals, coming from sensory receptors, somatosensorial fibers, the prefrontal cortex, limbic system and hippocampus can stimulate the hypothalamus to release CRH and AVP.[3]

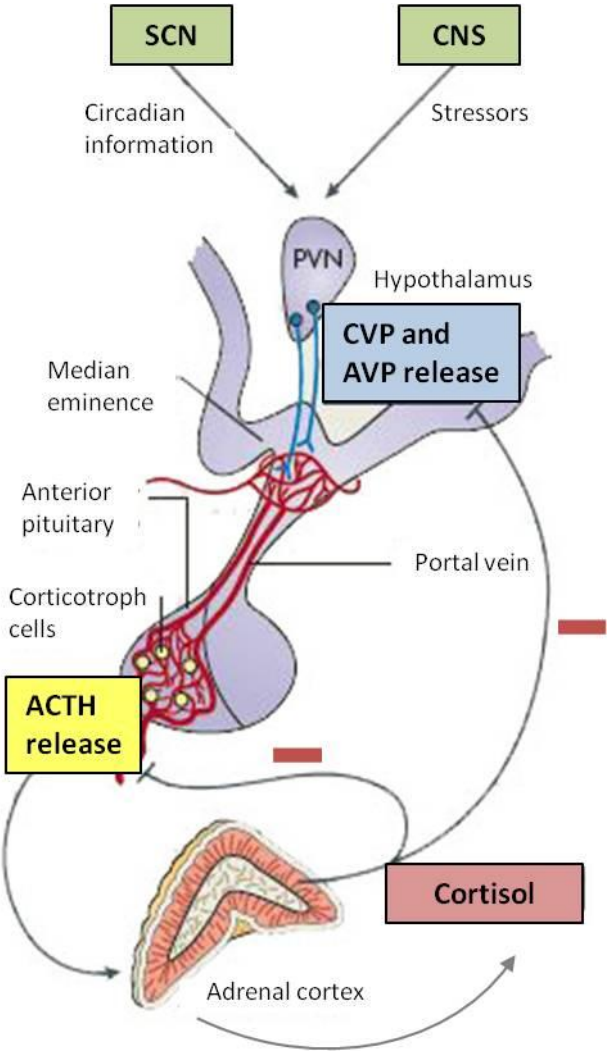

Figure 1 *- **Normal HPA-axis regulation***
with Supra-chiasmatic nucleus (SCN), central nervous system (CNS), paraventricular hypothalamus (PVH), corticotropin-releasing hormone (CRH), arginine vasopressine (AVP) and adrenocorticotropic hormone (ACTH). (Adapted from [4])

CRH neurons are located in the parvocellular division of the paraventricular hypothalamus (PVH) and release CRH at the median eminence into the primary capillary plexus of the hypothalamo-pituitary portal system. Furthermore, CRH is also expressed peripherally, with high levels found in the heart and placenta, but also in the limbic system, uterus, immune system, gastrointestinal tract, skin and adrenal gland. AVP is primarily produced in magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei, which project to the posterior pituitary to regulate vascular tonus and diuresis. However, AVP is also expressed by the CRH neurons in the PVN and acts as an auxiliary ACTH regulator in synergy with CRH.[3,5,6]

Regulation of the pituitary gland

ACTH is synthesized in the corticotroph cells of the anterior pituitary. It is derived from the pro-opiomelanocortin (POMC) gene, which after translation results in a prehormone. Several

posttranslational modification steps are required to process the POMC-prehormone in different peptides such as ACTH, β-lipotropin, which in turn gives rise to γ-lipoprotein and β-endorphin, and γ-melanocyte stimulating hormone (MSH). ACTH itself can be cleaved into α-MSH and corticotropin-like intermediate peptide (CLIP).[7]

After release of CRH into the hypothalamo-pituitary portal system, CRH reaches the pituitary where it binds to the CRH-receptor. There are two types of the CRH-receptors, but activation of the HPA-axis is mediated exclusively through the CRH-type 1 receptor. Binding to the receptor, which is G-protein coupled, causes activation of adenylate cyclase followed by an increase of cyclic AMP (cAMP) and protein kinase A (PKA). As such POMC gene transcription is stimulated. CRH stimulation leads to a biphasic response with the fast release from the pool of a pre-synthesized ACTH, and a slower sustained release of newly synthesized ACTH.[8] Furthermore, AVP, after binding to the vasopressine 1b-receptor (V1b-receptor) in the pituitary, can stimulate ACTH secretion weakly by itself, but synergizes with the effects of CRH on ACTH release.[3, 5, 6]

Next, a tightly regulated immune-neuroendocrine interface regulates the ACTH secretion. Pro-inflammatory cytokines, such as leukemia inhibitory factor (LIF), are known to stimulate POMC transcription and ACTH secretion. LIF is expressed in the corticotrophs of the pituitary gland and can act synergistic to CRH. Different other regulations of ACTH are described such as β-adrenergic catecholamines, vasoactive intestinal peptide and ghrelin which are all stimulators of ACTH, while opioids, oxytocin and atrial natriuretic peptide act as inhibitors of ACTH release. Furthermore, ACTH expression is negatively regulated by glucocorticoids.[7]

Feedback regulation by glucocorticoids

The HPA-axis is tightly regulated by a feedback system, where corticosteroids rapidly inhibit both CRH and ACTH. Glucocorticoids are lipid soluble and can therefore freely go through the blood-brain barrier. In the hypothalamus and pituitary they can bind to two receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR).[9] Glucocorticoids bind to the MR with a higher affinity than to the GR. As a consequence, both receptors respond to different levels of glucocorticoids. MRs are predominantly occupied under basal resting conditions, while GRs are occupied at the circadian peak and following stress. Furthermore, central MRs participate in control of the sensitivity of the neuroendocrine stress response system, while GRs are involved in termination of the stress response.[10]

The feedback regulation appears to work at three time frames.[11] Fast feedback, within seconds to minutes, cannot be exerted by influencing gene expression or protein synthesis, but likely works by inhibiting ACTH release.[12] Intermediate feedback occurs within 4 hours and inhibits hypothalamic CRH

synthesis. At last, slow feedback involves regulation of the pituitary ACTH content by decreasing levels of POMC mRNA. Therefore, slow feedback inhibits basal as well as stimulus-induced ACTH secretion. In addition, cortisol insensitive neural pathways could play a role.[13-15] Clearly, the feedback regulation of glucocorticoids is much more complex than the initially proposed simple closed loop feedback system.[16]

Circadian and ultradian rhythm

Besides the episodic release of cortisol in response to stress, cortisol is secreted continuously to maintain daily homeostasis. Both ACTH and cortisol follow a diurnal rhythm with maximum levels reached in the morning to anticipate wakening and daily activity, while minimal levels are observed during sleep. These levels fluctuate continuously and are influenced by food intake and physical activity. It was long assumed that this rhythm was driven by a CRH secretion rhythm. However, constant infusion of CRH restored the diurnal rhythm in CRH-knock-out mice suggesting that CRH, although necessary, is not the diurnal rhythm generator.[17] Recent evidence suggests that a biological clock exists to allow the human body to anticipate and prepare for changes and to ensure that different physiological processes are coordinated. The hypothalamic supra-chiasmatic nucleus (SCN) has been suggested for this role, as ablation leads to complete loss of circadian rhythm.[18] Recent evidence also strongly supports that besides the SCN as the central pacemaker, an integrated activity of the adrenal intrinsic oscillator and the splachnic nerves maintain the periodicity of cortisol.[19]

The diurnal rhythm is constructed by ultradian pulses that vary in amplitude and frequency during the day, with predominantly pulse amplitude determining the circadian rhythm. Increasing evidence supports the importance of this pulsatile release for maintaining transcriptional responsiveness of glucocorticoid-dependent genes and preventing desensitization of transcriptional responses in target tissues.[20-22] Feed-forward and feed-back interactions between ACTH and cortisol have been proposed to account for this ultradian rhythm.[23] Further research is needed to investigate the impact of circadian rhythm and pulsatility, since it was suggested to play a significant role in humans health and disease.[24]

1.1.2. Normal adrenal gland function

Adrenal gland anatomy and function

The adrenal glands are small retroperitoneal organs with a pyramidal structure, situated at the cranial pole of each kidney. (Figure 2 A) The gland consists of two parts, the cortex and medulla, which can be considered as two different organs based on their embryologic origin, function and regulation.[25, 26]

The medulla represents about 10% of the total adrenal gland and has a neuro-ectodermal origin. It is located in the center of the adrenal gland and consists of chromaffin cells that are responsible for

catecholamine production. The medulla is richly innervated by preganglionic sympathetic fibers and is therefore an extension of the sympathetic nervous system.

The adrenal cortex is derived from the mesoderm and organized in three concentric zones. These zones can be differentiated based on their morphological pattern and function. (Figure 2 B,C) Beneath the connective tissue capsule, the zona glomerulosa, consists of a thin region of columnar small cells arranged in spherical nests around the periphery of the cortex. It represents only 15% of the cortex. The intermediate layer, the zona fasciculata is responsible for 75% of the cortex and consists of large lipid-laden cells which form radial cords throughout a fibrovascular network. The third zone, the zona reticularis is composed of anastomosing cellular cords, which form a reticulum. Cells are lipid-sparse and contain often the brown pigment lipofuscin.[25, 26]

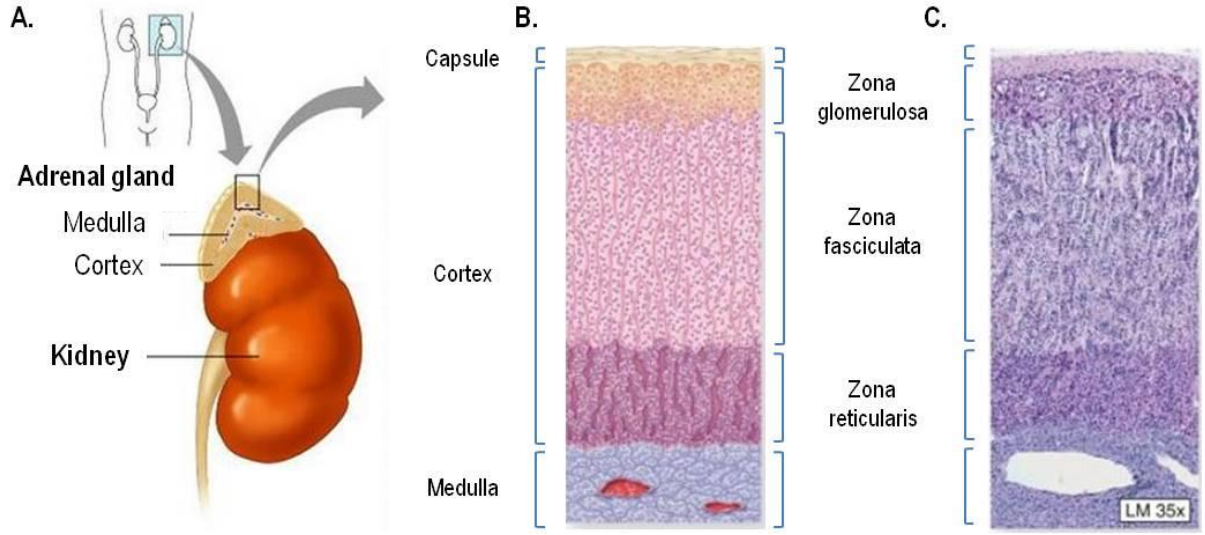

Figure 2** – **Adrenal gland anatomy and histology
***(A)** Schematic adrenal gland anatomy with transverse section of the adrenal gland. **(B)** Schematic drawing of a microscopic section of the adrenal gland. **(C)** Photograph of a hematoxylin-eosin-staining of the adrenal gland. (Adapted from Pearson Education Inc., Benjamin Cummings 2004 and McGraw Hill Companies Inc)*

The adrenal cortex produces three groups of steroid hormones. The zona glomerulosa secretes mineralocorticoids, predominantly aldosterone, which is responsible for the water and electrolyte balance in the body. The zona fasciculata secretes glucocorticoids, with cortisol being the most important one in humans. At last sex steroids are produced in the zona reticularis, which regulate sexual differentiation and function. In humans, this zone secretes predominantly the weak androgens androstenedione, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS). The function of these androgens is regarded as having little physiological significance when gonadal function is normal. The zonational difference in hormone secretion is explained by the specific and differential expression pattern of steroidogenic enzymes throughout the three zones.[25, 26]

Steroidogenesis

No hormones are stored in the adrenal gland and thus their secretion always requires an activation of the biosynthetic pathway. The production of steroid hormones in the adrenal gland starts with cholesterol by a process named steroidogenesis. The principal source of cholesterol (80%) is via uptake of low-density lipoprotein (LDL) cholesterol from the circulation via LDL-receptor (LDLR) mediated endocytosis. (Figure 3) To a lesser extent, both high-density lipoprotein (HDL) cholesterol and LDL are taken up in the adrenal gland with the scavenger receptor B1 (SCARB1) via selective cellular uptake.[27] Selective cellular uptake describes the selective uptake of only cholesterol-esters, without internalizing the complete lipoprotein particle itself. HDL-uptake is more important than previously thought, since patients with very low levels of LDL-cholesterol have a normal cortisol production.[28] The remaining 20% of cholesterol is generated *de novo* within the adrenal cortex, starting from acetyl coenzyme A (acetyl coA) and regulated by the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR). After uptake and production in the adrenal gland, the cholesterol molecules are esterified and stored in intracellular vesicles, where they reside until steroidogenesis starts.[29]

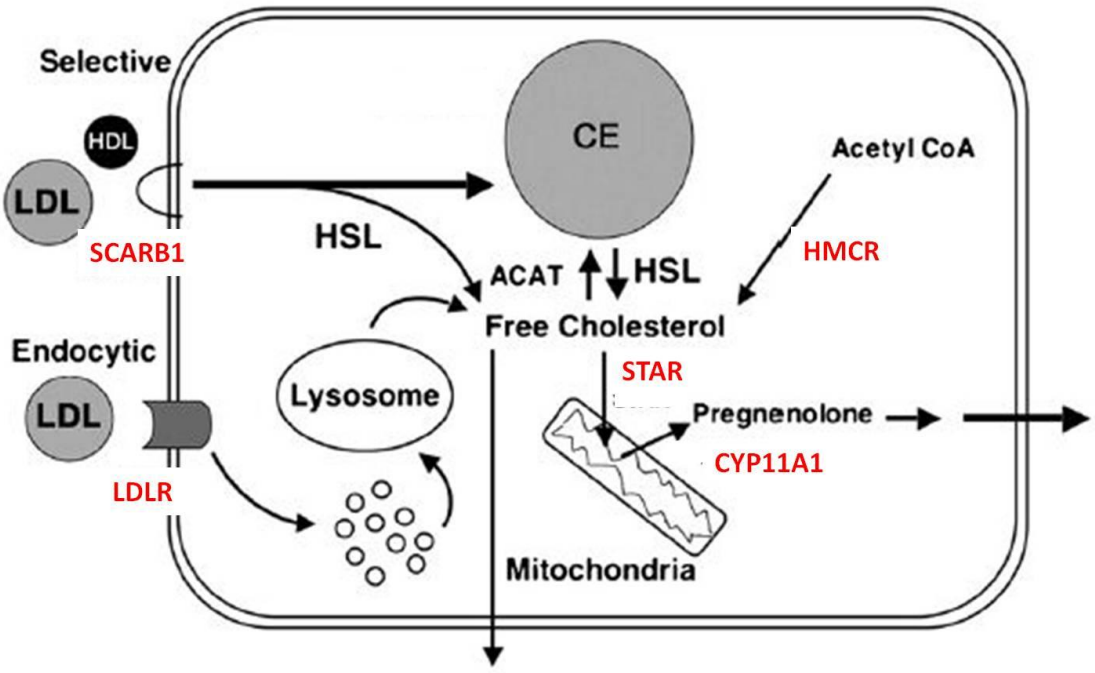

Figure 3- Cholesterol uptake and storage
with LDL-receptor (LDLR), scavenger receptor B1 (SCARB1), Hormone sensitive lipase (HSL), Acyl co-enzyme A: cholesterol acyltransferase (ACAT), cholesterol esters (CE), HMG co-enzyme A reductase (HMGCR), steroid acute regulatory protein (STAR) and cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1) (Adapted from [27])

Whenever cortisol is needed, cholesterol esterase (predominantly hormone sensitive lipase (HSL)) rapidly mobilizes cholesterol out of the lipid droplets to the cytoplasm. Thereafter cholesterol must be transported from the outer to the inner membrane of the mitochondria (IMM), where steroidogenesis starts. This transport is mediated by the steroidogenic acute regulatory protein (STAR), which is highly

important for cortisol production, since absence of this protein severely impairs steroidogenesis.[30] Furthermore, peripheral type benzodiazepine receptor (PBR) helps STAR to transport cholesterol to the mitochondria.[31]

In the IMM cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone, which is the rate-limiting step in steroidogenesis. (Figure 4) Next, pregnenolone is converted to progesterone in the endoplasmatic reticulum by 3β-hydroxysteroid dehydrogenase (3β-HSD), followed by a hydroxylation to 17OH-progesterone through the activity of 17α-hydroxylase (CYP17). Further hydroxylation by 21-hydroxylase (CYP21A2) results in 11-deoxycortisol. In the final step, which occurs again in the mitochondria, 11-deoxycortisol is hydroxylated by 11β-hydroxylase (CYP11B) to cortisol.[32]

The production of the other steroid hormones in the adrenal gland occurs in parallel and considerable interconversion between these pathways is possible. However, the expression of certain genes is restricted to specific cortical layers. As such the zona glomerulosa does not express CYP17 which is necessary for synthesis of 17-hydroxypregnenolone and 17-hydroxyprogesterone, while expression of aldosterone synthase (CYP11B2) is restricted to this zone.[33] (Figure 4)

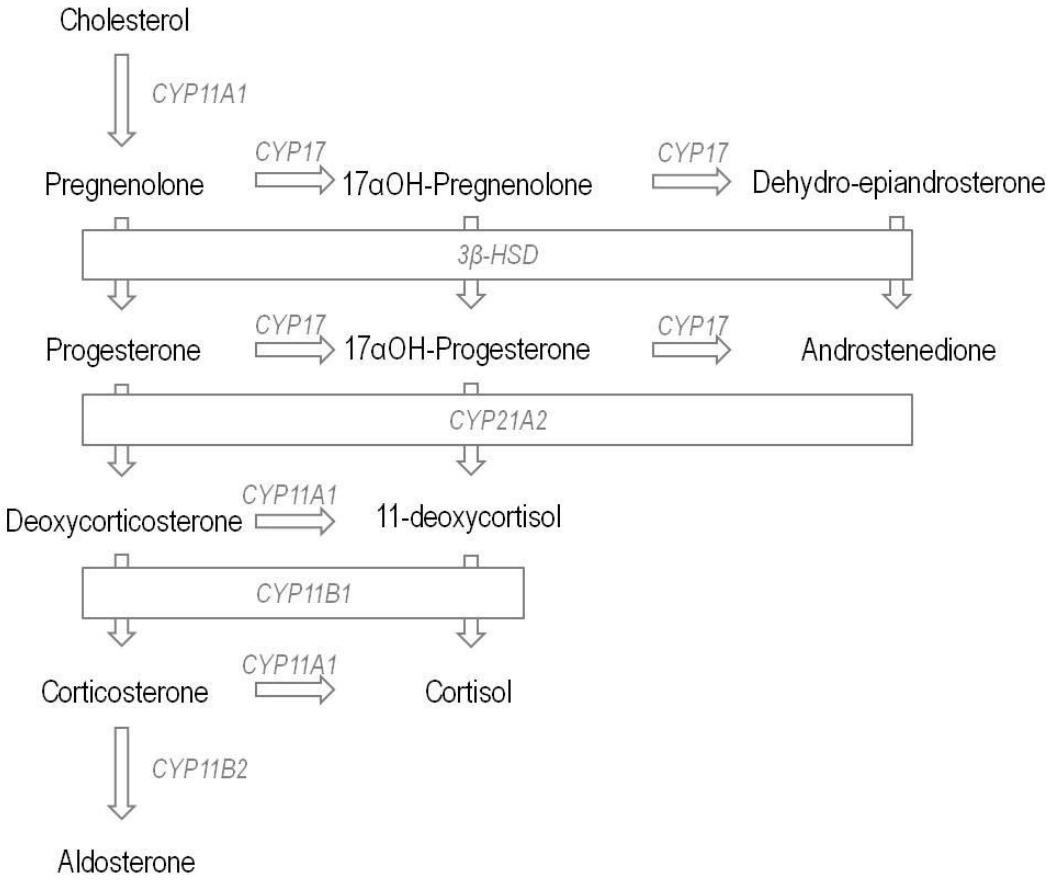

Figure 4 – The Steroidogenesis
with P450 cholesterol side-chain cleavage enzyme (CYP11A1), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase (CYP17), 21-hydroxylase (CYP21A2), 11β-hydroxylase (CYP11B)

Regulation by ACTH

The primary role of ACTH is to maintain the adrenal gland size, structure and function. Binding of ACTH to the melanocortin-2-receptor (MC2R) on the adrenocortical cells activates adenylyl cyclase and results in increased cAMP concentrations, which stimulates PKA.[34] PKA causes phosphorylation of different proteins and different nuclear transcription factors such as cAMP response element binding protein (CREB), which influences gene expression by binding to the cAMP response element (CRE) residing in the promoter regions of those genes.

ACTH function can be divided into acute and chronic effects. Immediately after ACTH is bound, phosphorylation of cholesterol esterase leads to the release of cholesterol from the lipid droplets.[35] Furthermore, rapid *de novo* protein synthesis of STAR is required for the transfer of cholesterol to CYP11A1. Consequently cortisol is released within minutes after ACTH secretion.[36] Different studies indicated that acute steroidogenesis is not dependent on new mRNA synthesis, but on the activation of several proteins or the synthesis of protein from pre-existing mRNA. Moreover, inhibition of phosphorylation impaired steroidogenesis.[36]

The chronic response to ACTH requires several hours and involves increased transcription and posttranscriptional regulation of genes encoding steroidogenic enzymes (CYPs) and thus enhances the synthetic capacity of the cells.[34] This regulation is complex, which is supported by the observation that no clear correlation exists between mRNA and protein levels.[37] ACTH phosphorylates different transcription factors for the steroidogenic genes among which the steroidogenic factor 1 (SF-1) is important.[38] In addition, ACTH increases the number of both the cholesterol uptake receptors (LDLR and SCARB1) as well as stimulates *de novo* production by upregulation of HMCGR, as such increasing adrenal cholesterol availability.[28, 29, 35] Furthermore, by stimulating cholesterol esterase while inhibiting acyl-CoA:cholesterol acyltransferase (ACAT) it promotes cholesterol delivery to the mitochondria.[39] ACTH also has a direct stimulatory effect on the expression of its own receptor in a dose-dependent manner.[40] Moreover, mRNA levels of MC2R accessory protein (MRAP), an essential component for MC2R function, are also increased after ACTH stimulation. By enhancing the expression of both MC2R and MRAP, the responsiveness of the adrenal cells to ACTH is amplified by its own stimulation.

Furthermore, ACTH stimulates adrenal growth by inducing cellular hypertrophy and hyperplasia. In the acute phase, ACTH causes hypertrophy of the adrenal cells, while hyperplasia only occurs if ACTH stimulus is maintained. Induction of the proliferation occurs via the extracellular signal-regulated kinases (ERKs) signaling pathway.[41] Both ACTH and growth factors activate this pathway, which is followed by phosphorylation of transcription factors that stimulate cell proliferation. Alternatively, ACTH also stimulates endothelin production and such influences adrenal blood flow.[42]

The multi-factorial role of ACTH in the adrenal gland was further demonstrated in POMC-deficient ($POMC^{-/-}$) mice. $POMC^{-/-}$ mice are incapable of producing ACTH because they lack the precursor molecule. Mutant mice showed no lipid staining on frozen sections of the adrenal glands. Furthermore gene expression of STAR and SCARB1 was decreased. Gene expression of CYP11A1, LDLR and HMGCR was unchanged or even increased, though corresponding protein levels were all decreased.[43] Furthermore, $POMC^{-/-}$ mice had smaller adrenal glands with loss of zonational structure.[44] All together these data further supports that ACTH is indispensible for adrenal gland function and structure.

Additional regulators

Increasing evidence supports the existence of non-ACTH dependent drivers of cortisol secretion. First, the adrenal cortex and medulla interact in two directions: The adrenal cortex can influence the synthesis of catecholamines in the medulla, while the cortex also receives postganglionic nerves from the medulla. The adrenal medulla consists of chromaffin cells, which receive splanchnic innervations. These medullar nerves are predominantly catecholaminergic and peptidergic and store dopamine, noradrenaline and adrenaline. Furthermore, the chromaffin cells also produce different neuropeptides, including opioid peptides, neuropeptide Y, vasoactive intestinal peptide and substance P, which also influence steroid production.[45] Furthermore, it is an oversimplification that medulla and cortex are two separate organs since chromaffin medullar cells are found in the adrenal cortex, allowing extensive contact zones for paracrine interaction.[26] Under non-stress conditions, splanchnic neural activity appears to be inhibitory, whereas during stress conditions neural activity is excitatory, explained by the difference in the released neurotransmitter.[46] The extensive innervations also sensitize the adrenal gland to ACTH, play a role in the diurnal output of the adrenal gland and stimulate growth of the adrenal gland.[47, 48]

The regulatory role of the immune system has been recognized already extensively. Pro-inflammatory cytokines have been shown to stimulate the HPA-axis at every level and can influence cortisol secretion independently of ACTH.[49-51] Different cytokines can also suppress the HPA-axis and GR function. Beside direct stimulation of the adrenal gland by circulating cytokines, these cytokines are also produced within the adrenal gland by immune cells and adrenocortical cells. Furthermore, cytokines also influence adrenal growth and differentiation with both stimulating as well as inhibiting effects being described.[45]

The adrenal medulla and intra-adrenal immune cells are also a source of extrahypothalamic CRH and extrapituitary ACTH, which could also account for the residual cortisol production without an increase in circulating ACTH levels.[45] Other intra-adrenal regulatory pathways have been suggested, such as a role

for blood flow and endothelial products, a role for growth factors, a role of the intra-adrenal renin-angiotensin system, though further research is needed.[52]

The question remains whether this extra-pituitary regulation can be an exclusive pathway or is only additional. It has been suggested that extra-pituitary regulation can be responsible for maintaining a basal small reserve, maintaining normal circadian rhythm fine tuning of secretion and compensatory growth. The role of this pathway in chronic forms of stress has been suggested, though needs to be explored.[45]

1.1.3 Cortisol function and metabolism

Cortisol transport

Immediately after cortisol is produced, it is released from the adrenal gland into the circulation. Being a steroid hormone, cortisol is relatively insoluble in plasma. Therefore, more than 95% of circulating cortisol is bound to proteins, predominantly to corticosteroid-binding globulin (CBG) and to a lesser extent to albumin. CBG has a high affinity for endogenous glucocorticoids, while affinity for exogenous glucocorticoids is less. Furthermore, CBG has a limited binding capacity and is saturated at a cortisol concentration of approximately 20-25 µg/dl indicating that circulating cortisol levels above this concentration increase the percentage of free cortisol exponentially. Albumin has a lower affinity for cortisol, but a higher binding capacity.[26]

Recent evidence suggests that CBG is more than just a transport protein.[53] It is secreted from the liver and follows a diurnal rhythm, opposite to the glucocorticoids diurnal rhythm. Since only free cortisol can be metabolized or cleared, low CBG levels also shorten the half-life of cortisol, and increase the plasma clearance and distribution volume of cortisol.[54] Furthermore, CBG affinity is affected by different factors such as pH, temperature and activity of neutrophils, as such regulating free cortisol levels. During inflammation, local elastases produced by neutrophils increase release of the glucocorticoid from CBG and therefore increase free cortisol. This is further emphasized by the loss of affinity caused by increased temperature.[53]

Importantly, routine cortisol assays measure total circulating cortisol which is determined by both free cortisol levels and protein-bound cortisol. However, only the unbound fraction is free to enter the target cells either to exert their metabolic effects or to be transformed into inactive metabolites. Currently, no commercial assays are available to measure circulating free cortisol levels. The gold standard to measure free cortisol is via ultra-filtration and equilibrium dialysis, yet these techniques are not available in most clinical settings and difficult to perform. As a practical alternative for direct measurement,

methods for estimating free cortisol levels are described and validated, with the Coolens equation being the most frequently used method. In this calculation, free cortisol levels are estimated based on total circulating cortisol levels and measured protein concentrations of CBG and albumin affinity.[55]

Cortisol signal transduction

When free cortisol, which is lipophylic, reaches the target organ, it rapidly diffuses across the cell membrane to enter the cytoplasm where it can bind to the GR. The GR is expressed in virtually all cell types. Cortisol can also bind to the MR with a 10-fold higher affinity. Though, MR expression is only restricted to certain tissues such as the kidney.[56]

The GR is a member of the nuclear receptor superfamily and functions as a ligand-dependent transcription factor. In the absence of ligands, the receptors are located in the cytoplasm and bound to a complex of proteins that stabilize the receptor in an inactive conformation. After binding of cortisol, the GR is released from the proteins and translocates to the nucleus where the GR can exert its effects which are complex and diverse. (Figure 5) In summary, to cause activation of gene expression the GR can bind directly after dimerization with another GR to a glucocorticoid response element (GRE) in the DNA or as a monomeric protein in cooperation with other transcription factor or by tethering other transcription factors to the DNA-bound monomeric GR.[56] Furthermore, GR can also repress gene transcription to a negative GRE via different ways.[56, 57] (Figure 5)

The observation that glucocorticoids also can induce effects within minutes, being too rapid to be the result of transcription, led to the hypothesis that glucocorticoids also have non-genomic effects. The non-genomic effects are diverse and increasing evidence suggests that second-messenger molecules and different membrane receptors are involved.[58]

Multiple GR isoforms are described and occur by alternative splicing. The GRα is the active form that binds ligands, while the GRβ does not bind glucocorticoids and acts as a dominant negative form by binding with GRα and preventing GR-dimerisation. The ratio of GRα to GRβ therefore reflects the glucocorticoid sensitivity of a cell. Furthermore, post-translational modification of the GRα is tissue specific and therefore regulates tissue specific glucocorticoid action.[59]

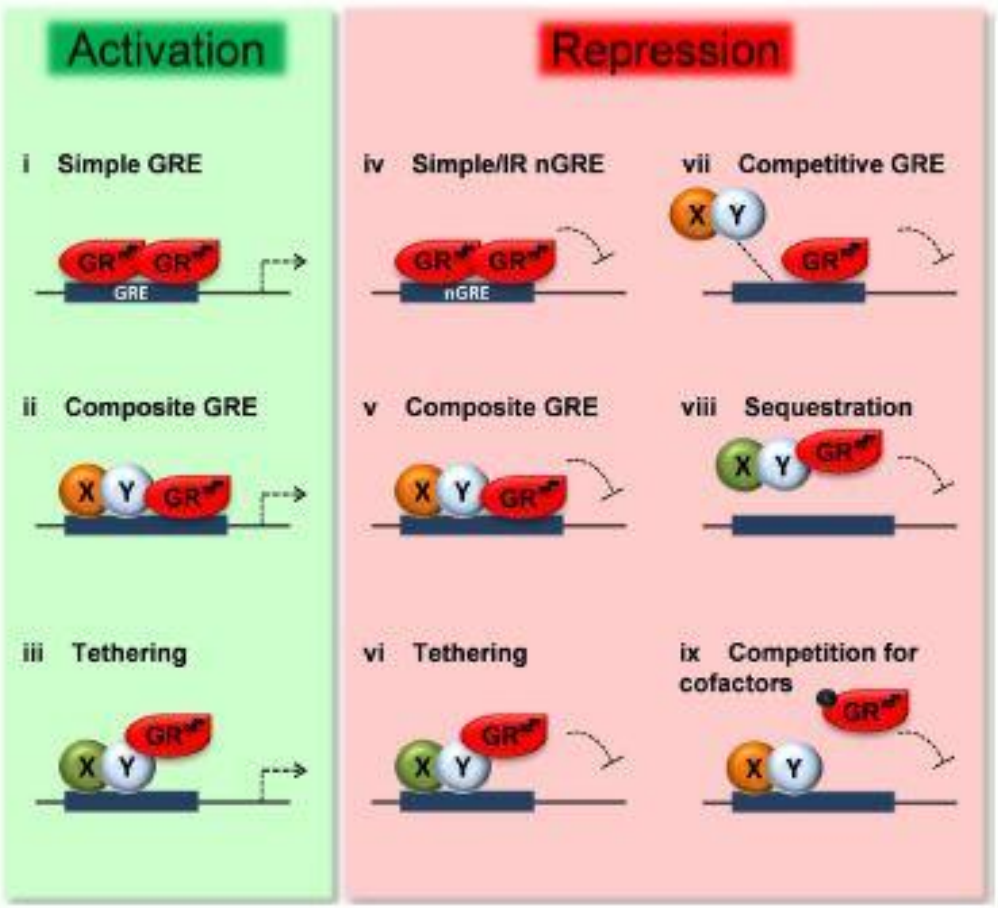

Figure 5 - GR Signaling causes activation or repression of gene transcription.
Left green panel: Activation is mediated by (i) binding of GR dimers to GRE, (ii) DNA binding of GR in concert with another transcription factor (XY), or (iii) binding of GR to a transcription factor by a tethering mechanism.
Right red panel: Repression is mainly achieved by (iv) direct binding of GR dimers to negative GRE (v) DNA-binding cross-talk with another transcription factor, (vi) interference of monomeric GR with the transactivation activity of transcription factors by a tethering mechanism, (vii) competition for an overlapping binding site (competitive GRE), (viii) sequestration of a DNA-bound transcription factor or (ix) competition for binding cofactors with other DNA-bound transcription factors. (From [56])

Cortisol function

The observation that GRs are found in almost all cells hints that cortisol has widespread effects. Indeed glucocorticoids are critical regulators of multiple fundamental processes, including metabolic homeostasis, cell proliferation and immunomodulation.[60, 61] It contributes to the provision of extra energy to vital organs by acutely shifting carbohydrate, fat and protein metabolism and by delaying anabolism. This comprises increasing glucose levels and insulin resistance, activating lipolysis, inhibiting protein synthesis and stimulating proteolysis.

Moreover, cortisol likely affects the hemodynamic system by intravascular fluid retention and by enhancing inotropic and vasopressor responses to respectively catecholamines and angiotensin II. In addition, glucocorticoids stimulate anti-inflammatory cytokine production and inhibit pro-inflammatory cytokines, cell migration and production of inflammatory mediators. These anti-inflammatory effects of

cortisol can be interpreted as an attempt to prevent overactivation of the inflammatory cascade. Furthermore, cortisol has diverse effects on bone, regulates cell growth and reproduction and influences the central nervous system by changing mood, food intake and behavior.[26] Despite the widespread positive acute effects, chronic hypercortisolism predominantly induced by long-term treatment causes severe side effects such as diabetes, osteoporosis, hypertension and muscle atrophy.

Cortisol metabolism

The metabolism of cortisol is complex and differs within tissues. (Figure 6) Cortisol can be inactivated to cortisone via 11β-hydroxysteroid dehydrogenase (11β-HSD) type-2 mainly in kidney. However, other mineralocorticoid target tissues also express this enzyme such as placenta, gastro-intestinal tract, the salivary glands and lungs. Cortisol can also be regenerated from cortisone via 11β-HSD type-1. This enzyme can function in the two directions and can therefore also inactivate cortisol. The directionality is tissue specific and depends upon the availability of the co-factor NADPH. 11β-HSD type-1 is widely distributed in the body including liver, adipose tissue, gonads, bone, muscle and skin, all glucocorticoid target tissues.[62] Both 11β-HSDs play a pivotal role in cortisol function at tissue level by influencing the availability of active cortisol. Only cortisol can bind to the GR and MR and exert its function. Therefore, conversion into cortisone inhibits binding to the receptor, which is predominantly of importance in kidney to prevent aldosterone-like effects by binding to the MR.[62]

The principle route of irreversible cortisol breakdown is via the A-ring reductases, 5α-reductase and 5β-reductase, which mediate the rate-limiting step in cortisol breakdown. This results in dihydro-metabolites, which are further reduced by 3α-HSD to form the tetrahydro-metabolites. Cortisol breakdown via 5α-reductase occurs predominantly in liver and adipose tissue and leads to the formation of the metabolite 5α-tetrahydrocortisol (allo-THF). Two genes of the steroid α-reductase family (SRD5A) encoding for 5α-reductase are described. Type 1 is predominantly expressed in androgen-independent tissue, such as liver, adipose tissue, skin and brain, while type 2 predominates in androgen dependent tissues such as the prostate where it activates testosterone.[63]

Also present in the liver, 5β-reductase degrades both cortisol and cortisone into 5β-tetrahydrocortisol (THF) and tetra-hydrocortisone (THE) respectively. This enzyme is encoded by the aldo-keto-reductase member D1 (AKR1D1) gene. Furthermore, 5β-reductase plays an essential role in bile acid synthesis, supported by the observation that patients with a point mutation in the 5β-reductase gene (AKR1D1) have low levels of cholic acids and chenodexoycholic acids.[64]

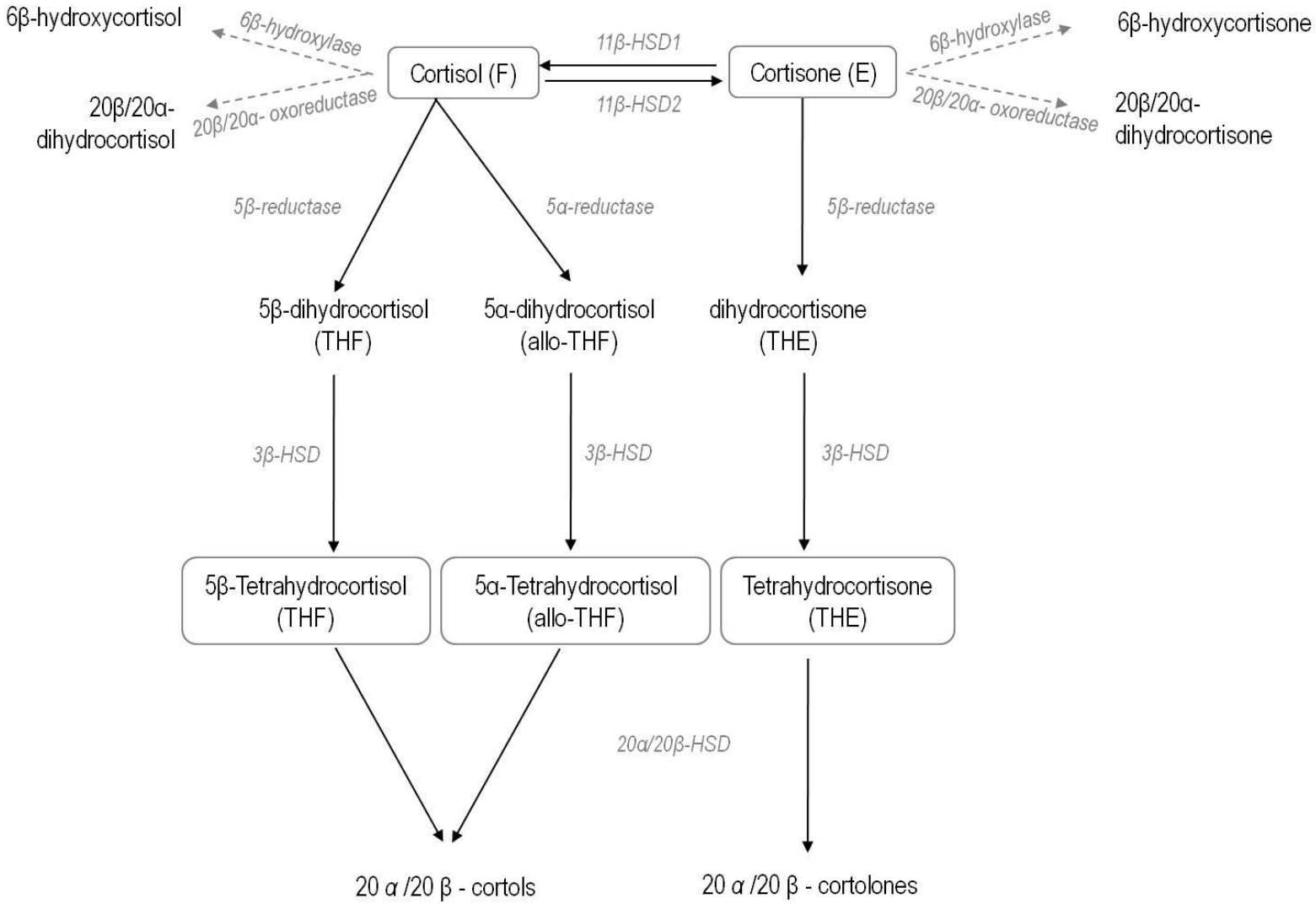

Figure 6 – Schematic overview of cortisol metabolism

with the major metabolites depicted in boxes.

Other metabolizing pathways consist of 6β-hydroxylation of cortisol, the reduction of the 20-oxogroups or conversion of the tetrahydro-metabolites into cortols and cortolones.[65] Afterwards, conjugation with either sulfates or glucuronides enhances solubility of the metabolites and therefore the renal secretion. Only 1% of circulating cortisol is excreted unaltered in the urine, while the metabolites of the A-ring reductases predominate.[66]

Since all the metabolites are excreted in urine, enzyme activity is mostly assessed by measuring the ratios of the different urinary cortisol metabolites. 5α-Reductase activity is estimated by the allo-THF/F ratio and 5β-reductase activity by THF/F and THE/E ratios. The activity of renal 11β-HSD2 was estimated by the E/F ratio. The ratio (allo-THF+THF)/THE only reflects the balance of cortisol/cortisone interconversion.[67, 68] However, investigation with specific 11β-HSD-inhibitors showed that urinary metabolites are not appropriate to investigate their activities and infusion of isotopically labeled cortisol, generating labeled metabolites, is now the golden standard to quantify 11β-HSD activity.[69]

Regulation of these enzymes is complex and incompletely understood due to conflicting results. Furthermore, the clinical significance of the suggested regulations needs to be elucidated. Growth hormone and insuline growth factor-1 were described to inhibit 11β-HSD1, but to stimulate 5α-reductase. Pro-inflammatory cytokines stimulate the expression and activity of 11β-HSD1. Glucocorticoids regulate both 11β-HSD1 and 2, although conflicting data exist. Insulin has been described either to inhibit or not affect 11β-HSD1 activity while it could stimulate 5α-reductase. Luteinizing hormone and follicle stimulating hormone stimulate, while progesterone inhibits 11β-HSD1. Thyroid hormones inhibit 11β-HSD1 and stimulate 5α-reductase.[26, 62, 67] Interestingly, bile acids, both conjugated and unconjugated, are potent inhibitors of the cortisol metabolizing enzymes, via competitive inhibition at low-physiological concentrations and by suppression of gene and protein expression at elevated concentrations.[70-72] Furthermore, different diseases can influence cortisol metabolism such as thyroid disorders, kidney diseases, and obesity.

1.2 STRESS RESPONSE IN CRITICAL ILLNESS

1.2.1 Critical illness

Critical illness is defined as any life-threatening condition requiring support of vital organ functions to prevent imminent death. This condition can be evoked by a variety of insults such as multiple trauma, complicated surgery and severe medical illnesses. Without modern critical care medicine, critically ill patients would not survive.

Although survival from previously lethal conditions is nowadays possible, often recovery does not swiftly follow. Then, patients enter a chronic phase of critical illness during which they continue to depend upon vital organ support for weeks, while the original trigger of the critical illness has long been resolved. This stage is characterized by distinct endocrine and metabolic alterations which may no longer be solely beneficial as they may hamper recovery. An example is the relative maintenance of fat stores while large amounts of proteins continue to be wasted from skeletal muscle and other organs.[73] This response may impair recovery of vital organ functions, extend weakness and hamper rehabilitation[74] and expose patients to severe, often infectious, complications.[75] The understanding of the mechanisms determining why certain patients recover and others don't remains very limited, but recent studies point to variable abilities to remove cell damage as playing a key role.[76, 77] When patients remain dependent upon critical care support, it is ultimately decided to withdraw care because of futility. Hence, further understanding the underlying pathways of recovery and investigating whether these pathways can be beneficially affected by treatment is of high clinical relevance.

1.2.2 HPA-axis regulation in critical illness

Critical illness is the ultimate form of severe physical stress and all stress responses are expected to be of greater magnitude in critically ill patients. As part of this stress response, elevated plasma concentrations of the stress hormone cortisol hallmark critical illness. The more severe the critical illness in human patients, and thus the higher the risk of death, the higher plasma cortisol concentrations appear to be.[78] However, also inappropriately low plasma cortisol has been linked to increased mortality Moreover, circulating levels of DHEAS and aldosterone are low during both the acute and prolonged phase of critical illness. Traditionally, this high cortisol levels were attributed primarily to the stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis with a shift in production away from both mineralocorticoid and adrenal androgens towards glucocorticoid production.[79]

Beside high total cortisol levels, increased free cortisol levels are also observed during critical illness, explained by decreased CBG and albumin levels.[80] This hypoproteinemia could be explained by hepatic failure or could be induced by high glucocorticoid levels, known to increase CBG clearance. Furthermore, sepsis is characterized by a reduced activity and amount of CBG levels. CBG affinity is also decreased at the site of infection caused by increased elastase activity from the neutrophils and increased temperature, increasing local cortisol availability.[80-84]

The biological response of cortisol is not only mediated by the free cortisol concentrations, also the GR function plays a role. Increasing evidence from both animal and human experiments suggests that GR availability, GR affinity and GR translocation are regulated during critical illness.[85-90] Decreased GR

affinity was observed in different animal and *in vitro* studies and confirmed in human studies of patients with chronic inflammation. GR expression was shown to be reduced in white blood cells of critically ill patients.[90] However, the expression of GR in different tissues warrants more research in critically ill patients. Furthermore, the clinical relevance of these cellular changes needs to be elucidated.

High cortisol levels are traditionally attributed to an activation of the HPA-axis and therefore to increased cortisol production. This conclusion is based on the observation of increased circulating cortisol levels and on the general concept of the stress response. However, plasma hormone concentrations are not only influenced by hormone secretion, but are the net result of hormone secretion, distribution in plasma, binding to plasma proteins and plasma clearance.[91] Prior to this PhD project, direct investigation of the cortisol production and clearance during critical illness was lacking.

Published data on plasma ACTH concentrations in critically ill patients are scarce. This lack of data is likely explained by the essential requirements for correctly assaying plasma ACTH: blood samples should be placed on ice and spun cold prior to assay or prior to freezing plasma for later assay. Already in 1995, however, Vermes *et al.* observed that plasma ACTH concentrations in patients suffering from severe trauma or sepsis were only transiently elevated when compared to healthy control values, and dropped after a few days to values below the healthy lower limits of normality.[92] Other studies investigated ACTH after surgery or only at one time point, which hampers general conclusions on ACTH during critical illness.[93-95]

Low plasma ACTH in the presence of high plasma cortisol concentrations have been interpreted as non-ACTH driven cortisol production.[96] (Figure 7) Beside alternative simulators of the adrenal gland or increased ACTH sensitivity, this constellation could be caused by reduced cortisol breakdown suppressing the production of adrenocortical hormones via feedback inhibition. This mechanism was already suggested 50 years ago. However, prior to this PhD project, this possibility was not further explored.[97, 98]

The studies that investigated the interaction between ACTH and cortisol during critical illness were based on one daily time point.[93-95] Considering the fact that both ACTH and cortisol are secreted in pulses, one could easily miss the link between two pulses with only one time point.[96]

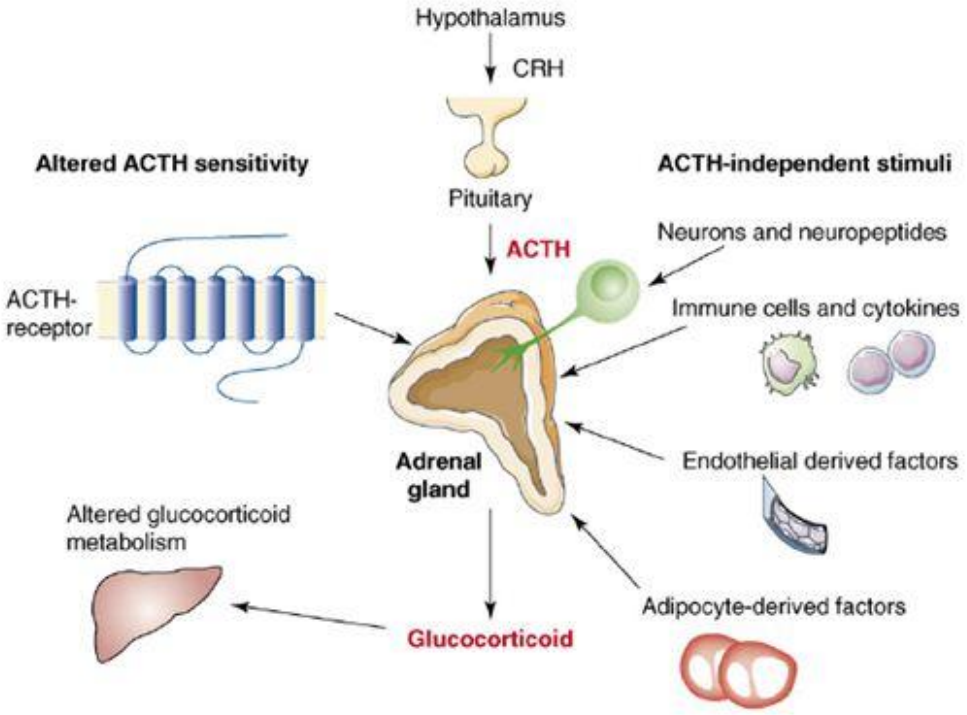

Figure 7 – non-ACTH dependent mechanisms of the ACTH-cortisol dissocation. *(from* [96])

1.3 ADRENAL INSUFFICIENCY

An intact HPA-axis is indispensable to survive critical illness. This was evidenced by animal experiments showing that mortality of sepsis is much higher after adrenalectomy.[99] Yet, prolonged critical ill patients often appear to suffer from a failing HPA-axis, which is lethal when left untreated. Therefore, recognition and appropriate treatment of this condition is crucial.[100]

1.3.1 Absolute adrenal insufficiency

Etiology

According to the underlying mechanism, absolute adrenal insufficiency, also known as Addison disease, can be divided into primary, secondary and tertiary causes. Primary adrenal insufficiency is caused by diseases of the adrenal gland itself. Lack of ACTH function or secretion due to pituitary abnormalities is labeled as secondary adrenal insufficiency, while insufficient AVP and CRH secretion and function can cause tertiary adrenal insufficiency.[101] Long-term, high-dose exogenous glucocorticoid therapy is the most common cause of tertiary adrenal insufficiency due to suppression of the HPA axis by exogenous glucocorticoid.[100-102]

Diagnosis

Adrenal insufficiency often has an insidious onset which is often missed and therefore adrenal insufficiency can evolve into an adrenal crisis evoked by an intercurrent illness. An adrenal crisis should always be ruled out in any patient with cardiovascular shock that is resistant to vasopressors. An acute adrenal insufficiency can also be initiated by acute adrenal hemorrhage, which is clinically presented by hypotension, abdominal pain, falling hemoglobin and progressive hyperkalemia. The combination with other non-specific signs of chronic adrenal failure can hint to acute adrenal insufficiency.[100-102]

Chronic adrenal insufficiency is characterized by non-specific symptoms such as fatigue, (orthostatic) hypotension, weight loss, abdominal cramps, nausea, anorexia, and diarrhea. The most specific sign of primary adrenal insufficiency is hyperpigmentation of the skin and mucosa and craving for salt. Furthermore, laboratory analysis can reveal normocytic anemia, lymphocytosis, eosinophilia, metabolic acidosis, hypercalcemia and hypoglycemia. Signs of mineralocorticoid deficiency (hyponatremia and hyperkalemia) are only present in case of primary adrenal deficiency.[101, 102]

Clinical suspicion of adrenal insufficiency can be confirmed by the presence of low baseline cortisol levels (<3µg/dl in the morning). Circulating ACTH levels should be measured to differentiate between primary and secondary failure with low ACTH levels being indicative of secondary adrenal insufficiency, while high ACTH levels point to a primary cause. Furthermore, circulating levels of renin and aldosterone add to the differentiation of primary and secondary failure. The ACTH-stimulation test confirms the diagnosis. Therefore, 250 µg of synthetic ACTH is given and the circulating cortisol levels at baseline and at 30 and 60 minutes after injection represents the adrenal ability to produce cortisol. Cortisol levels <20µg/dl after ACTH simulation are indicative of adrenal insufficiency. The use of a low dose ACTH-stimulation test is suggested to increase sensitivity in patients with mild adrenal insufficiency. Other diagnostic tests such as the insulin tolerance test, the metapyrone test, the CRH stimulation test and adrenal imaging could add to the differential diagnosis.[100, 103]

Treatment

Treatment of chronic adrenal insufficiency consists of substitution of the daily cortisol production by exogenous glucocorticoids. The current guidelines recommend 10-12 mg/m² hydrocortisone, given in two or three doses with a higher dose in the morning to mimic the circadian endogenous production. In primary adrenal failure, mineralocorticoids should be added, while the substitution of androgens is less clear. For all patients the use of stress-doses to prevent adrenal crisis should be implemented when a new major stressor such as surgery is suspected and patients should be educated to be aware of this risk.[103]

An adrenal crisis is life-threatening and therefore, immediate therapy should be initiated when clinically suspected. This treatment consists of reversal of hypotension and electrolyte disorders, combined with high dose glucocorticoid substitution (200 mg hydrocortisone per day) until the patient's condition is stable, and followed by a tapering down of the dose.

Absolute adrenal insufficiency and critical illness

In critical illness, absolute adrenal insufficiency can concomitantly be present. Adrenal insufficiency should be suspected when unexplained catecholamine-resistant hypotension is present, especially when combined with concurrent hyperpigmentation, hyponatremia or hyperkalemia. In addition, spontaneous adrenal insufficiency due to adrenal hemorrhage and adrenal-vein thrombosis occurs more frequently due to coagulation disorders related to critical illness. Furthermore, different medications often used in the intensive care unit (ICU) can affect cortisol production. Etomidate is known to inhibit cortisol production, even after a single dose. Other drugs know to influence the HPA axis are predominantly azoles, anticoagulants, phenobarbital, phenytoin, rifampin, opioids, chlorpromazine and imipramine.[104]

It is generally accepted that patients with an established diagnosis of primary or central adrenal failure or patients on chronic treatment with systemic glucocorticoids prior to critical illness should receive additional coverage to cope with the acute stress.[105, 106] Also, patients who are diagnosed with an acute Addisonian crisis in the ICU are typically treated with high doses of glucocorticoids. This therapeutic strategy is based on the assumption that cortisol production is several-fold increased in critical illness. The conventional treatment proposes the administration of a bolus of 100 mg of hydrocortisone followed by 50 to 100 mg every 6 hours on the first day, 50 mg every 6 hours on the second day, and 25 mg every 6 hours on the third day, tapering to a maintenance dose by the fourth to fifth day.[105, 106]

1.3.2 Relative adrenal insufficiency

Activation of the HPA-axis is assumed to be sometimes insufficient to cope with severe illness and to cover the needs for cortisol to survive. As plasma cortisol in this condition is still higher than during health, it has been labeled 'relative adrenal insufficiency' (RAI),[107] or more recently, 'critical illness-related corticosteroid insufficiency' (CIRCI).[108] Both terms comprise the inability to raise adrenal steroid production up to the level required to match the level of stress. Such 'relative failure' could be due to a malfunctioning at any level of the HPA-axis and/or to resistance of the peripheral tissues to cortisol during critical illness.[108, 109] Depending on the diagnostic criteria, the incidence varies widely. However, septic patients and patients with acute respiratory distress syndrome are more prone to RAI.

Furthermore, liver diseases and adrenal disorders frequently coincide, which led to the introduction of the term “hepato-adrenal syndrome”.[110]

Pathophysiology

In 1946 Hans Selye suggested that ‘exhaustion’ of the adrenal cortex could occur. However, the underlying mechanisms still remain unknown. Pro-inflammatory cytokines are suggested to induce tissue resistance or inhibit ACTH function. Furthermore, impaired blood supply to the pituitary can induce ischemia or necrosis, which is followed by the accumulation of nitric oxide or central neuropeptides, leading to decreased hormone secretion.[101] Additionally, different neuropeptides, oxidative stress, altered adrenal blood flow, substrate deficiency due to low circulating cholesterol or interfering medications are suggested to play a role.[104] Since cholesterol production is exerted by the liver, patients with liver disease have defective cholesterol production, which increases the risk of adrenal insufficiency. Furthermore, the incidence of coagulopathy is higher in these patients leading more frequently to adrenal hemorrhage.[110] Tissue resistance, the second part of the definition of CIRCI, can be explained by decreased glucocorticoid delivery or decreased glucocorticoid action. As explained before, due to low CBG levels and decreased CBG affinity, cortisol transport is impaired and GR receptor levels and affinity are affected during critical illness.

Diagnosis

Initial diagnosis should always be based on clinical suspicion. Hypotension refractory to fluid resuscitation or vasopressors is indicative of CIRCI with or without non-specific symptoms of adrenal failure as described previously.

Suggested diagnostic criteria for CIRCI in critically ill patients were based on a landmark study by Annane *et al.* who identified a plasma cortisol incremental response of <9 µg/dl after injection of 250 µg ACTH and a high baseline cortisol level (>34 µg/dl) as most discriminative for increased risk of death.[107] However, other investigators have not all been able to replicate these findings and until now no consensus has been reached. Furthermore, also the dose of the stimulation test remains controversial. A dose of 250 µg of ACTH leads to supra-physiologic ACTH levels and could therefore overcome ACTH resistance. Alternatively, a 1 µg stimulation dose was suggested. However, this has not been extensively studied in critically ill patients and this test has not been clinically implemented in the critical care setting, due to conflicting results.

Alternatively, a random total cortisol of <10µg/dl during critical illness has been suggested for the diagnosis of CIRCI.[108] However, total plasma cortisol concentration is the net result of adrenal

production and secretion, distribution, binding and elimination of cortisol. Also, cortisol is secreted in a pulsatile manner.[111] It thus seems unlikely that one could judge the adequacy of the adrenal cortisol production in response to critical illness merely by a single measurement of total plasma cortisol. Furthermore, total plasma cortisol concentrations do not reflect glucocorticoid signaling. First, only free cortisol can pass the cell membrane to exert its function by binding to the GR. Critical illness acutely suppresses circulating levels of the binding proteins, CBG and albumin, and also alters CBG binding affinity via increased cleavage from CBG at inflammatory loci or by increased temperature.[80-84] Hence plasma free cortisol may be more appropriate than total cortisol to assess HPA-axis function. However, plasma free cortisol assays are currently not readily available and normal ranges for plasma free cortisol during critical illness have not been defined. Additionally, increasing evidence from both animal and human experiments suggests that GR availability in different tissues, the GR affinity and translocation are regulated during critical illness.[85-90] In septic patients, for example, the dominant negative β-isoform of the GR was induced from admission, which downregulates glucocorticoid action.[86] Although these changes could be adaptive or maladaptive, they preclude conclusions about 'adequacy' of cortisol availability and function during illness. Finally, assays that are commonly used to quantify plasma cortisol concentrations are often inaccurate and very substantially[112] indicating that it is virtually impossible to define CIRCI based on one cut-off value in clinical practice without better standardizing the methodology. It may be necessary to use mass spectrometry for this purpose.[113, 114]

It was recently suggested that measuring interstitial cortisol levels could be interesting to assess the amount of active tissue cortisol levels in critically ill patients.[115, 116] For this purpose, a microdialysis catheter is inserted into the subcutaneous adipose tissue. However, edema is frequently present and regional blood flow varies in critically ill patients.[117] Hence, the benefit of this invasive technique could be questioned.

Recent guidelines from the surviving sepsis campaign now no longer recommend the use of the ACTH stimulation test to diagnose CIRCI.[118] They suggest to use a random cortisol level <18 µg/dl in a patient with septic shock and clinical suspicion of adrenal failure as an indication for initiation of steroid therapy.[118]

Treatment

Although the concept of CIRCI is widely accepted, it remains highly debated whether or not it should be treated with exogenous glucocorticoids and if so, with which doses. Indeed, randomized controlled studies generated conflicting results. The first large trial by Annane *et al.*[119] investigated 300 patients with septic shock on vasopressor therapy. All patients were randomly assigned to be treated with

placebo or 200 mg hydrocortisone plus 50 µg fludrocortisone. In all patients an ACTH stimulation test was performed. They observed that the glucocorticoid treatment reduced the duration of vasopressor therapy and caused a 10% absolute risk reduction of 28 days-mortality in those patients who did not respond sufficiently to the ACTH stimulation test. However, the study was criticized based on different methodological aspects, such as the use of etomidate and statistical issues.

To settle controversy, a new large multi-center randomized-controlled double-blind trial investigated 499 patients to evaluate the impact on 28 days-mortality of placebo versus 200 mg hydrocortisone daily for 5 days after which a tapering period followed.[120] This study confirmed that hydrocortisone treatment increased shock reversal in all patients. However, hydrocortisone did not improve survival, neither in the non-responders to ACTH stimulation, nor in the entire population. Moreover, an increased incidence of septic shock relapse was observed. Again, concerns were raised since the study was underpowered, weakening the overall conclusions. Since then no adequately designed study was performed to resolve the remaining questions.

In view of the controversy, recent guidelines from the surviving sepsis campaign now recommend, although with a weak level of recommendation, to only treat patients with hypotension refractory to vasopressor or fluid resuscitation. A dose of 200 mg hydrocortisone via continuous infusion is desirable, with a quick tapering down of the dose whenever vasopressors are no longer needed. Furthermore, they do not advise glucocorticoid treatment for patients with sepsis without shock.[118]

1.4 REMAINING QUESTIONS

Despite the general agreement that adequate HPA-axis function is crucial for survival in critical illness, important lack of knowledge currently retains further improvement of both diagnosis and treatment of adrenal failure in critical illness. Moreover, even the fundamentals of cortisol and ACTH regulation during critical illness are not fully understood. It is imperative that we explore ACTH and cortisol secretion during critical illness to gain more insight in the so-called ACTH-cortisol dissociation. Despite different suggestions in the literature, research is lacking to explain this dissociation. A better understanding of the underlying pathophysiology will allow us to better refine subgroups that may benefit from glucocorticoid therapy and increase survival.

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CHAPTER 2

AIM AND STUDY OBJECTIVES

AIM

The main objective of this doctoral thesis was to explore the regulation of cortisol secretion and metabolism during critical illness in order to gain more insight in the pathophysiology of adrenal insufficiency that occurs in ICU patients.

GENERAL HYPOTHESIS

The general hypothesis of this doctoral thesis postulates that during critical illness reduced cortisol breakdown, rather than continuously stimulated cortisol secretion, contributes to maintaining the elevated plasma cortisol concentrations. Negative feedback inhibition, exerted by high circulating cortisol levels would then explain low ACTH levels. When ACTH deprivation sustains, adrenal integrity and function could be negatively affected, predominantly in the prolonged phase of critical illness.

SPECIFIC STUDY OBJECTIVES

This hypothesis was tested by means of the following specific study objectives:

(1) To investigate cortisol production and breakdown in relation to circulating ACTH and cortisol levels via 5 clinical studies in which blood, urine and tissue samples of critically ill patients and matched healthy controls are compared. Therefore, we determined (i) daily ACTH and cortisol levels during the first week of ICU stay; (ii) plasma cortisol clearance, breakdown and production during deuterated-steroid tracer infusions; (iii) plasma clearance of 100 mg hydrocortisone; (iv) urinary cortisol metabolites and (v) mRNA and protein expression in liver and adipose tissue to assess the major cortisol-metabolizing enzymes.

(2) To study ACTH and cortisol hormonal secretory dynamics during critical illness, and how both are interrelated, via mathematical analyses of repeated sampling time-series obtained from patients and matched healthy control subjects. Besides other methodologies (Approximate entropy, CrossApproximate entropy and the dose response relationship), deconvolution analysis was used to derive secretion profiles from the plasma concentration profiles.

(3) To identify the impact of sustained ACTH deprivation on the adrenal glands during critical illness. Since ACTH exerts a profound trophic effect on the adrenal gland and its steroidogenic potential, adrenal glands were harvested within 24h after death from ICU-non-survivors and from sudden out-of-hospital deaths, to investigate these key ACTH effects.

CHAPTER 3

REDUCED CORTISOL METABOLISM DURING CRITICAL ILLNESS

<u>Adapted from:</u> Boonen E, Vervenne H, Meersseman Ph, Andrew R, Mortier L, Declercq PE, Vanwijngaerden YM, Spriet I, Wouters PJ, Vander Perre S, Langouche L, Vanhorebeek I, Walker BR, Van den Berghe G. Reduced cortisol metabolism during critical illness. N Engl J Med 2013;368(16):1477-1488

3.1 ABSTRACT

Background: Critical illness is often accompanied by hypercortisolemia, which has been attributed to stress-induced activation of the HPA-axis. However, low ACTH levels have also been reported, which may be due to reduced cortisol metabolism.

Methods: In a total of 158 patients in the intensive care unit and 64 matched controls, we tested five aspects of cortisol metabolism: daily ACTH and cortisol levels; plasma cortisol clearance, metabolism and production during infusion of deuterium-labeled steroid hormones as tracers; plasma clearance of 100 mg of hydrocortisone; levels of urinary cortisol metabolites and levels of messenger RNA and protein in liver and adipose tissue, to assess the major cortisol-metabolizing enzymes.

Results: Total and free circulating cortisol levels were consistently higher in patients than in controls, whereas ACTH levels were lower ($P<0.001$ for both comparisons). Cortisol production was 83% higher in the patients ($P=0.02$). There was a reduction of more than 50% in cortisol clearance during tracer infusion and after the administration of 100 mg of hydrocortisone ($P\leq0.03$ for both comparisons). All these factors accounted for an increase by a factor of 3.5 in plasma cortisol levels in patients, as compared with controls ($P<0.001$). Impaired cortisol clearance also correlated with lower cortisol response to ACTH stimulation. Reduced cortisol metabolism was associated with reduced inactivation of cortisol in the liver and kidneys, as suggested by urinary steroid ratios, tracer kinetics and assessment of liver-biopsy samples ($P\leq0.004$ for all comparisons).

Conclusions: During critical illness, reduced cortisol breakdown, related to suppressed expression and activity of cortisol-metabolizing enzymes, contributed to hypercortisolemia and hence ACTH suppression. The diagnostic and therapeutic implications for critically ill patients are unknown.

3.2 INTRODUCTION

Critical illness, an example of severe acute physical stress, is often accompanied by hypercortisolemia that is proportionate to the severity of illness.[1,2] This observation has traditionally been attributed to stress-induced activation of HPA-axis and increased ACTH-driven cortisol production.[3] However, this stress response may not be sufficient for a good prognosis in patients with relative adrenal insufficiency.[4-7] Moreover, Vermes *et al.*[8] reported only transiently elevated levels of ACTH during critical illness, whereas cortisol remained high, a paradoxical dissociation between cortisol and ACTH levels that has also been observed in other stress conditions.[9]

In addition to alternative activators of cortisol production, such as proinflammatory cytokines,[9,10] another explanation for hypercortisolemia in the presence of suppressed ACTH could be reduced cortisol removal. The principal routes of cortisol clearance occur in the liver (through A-ring reductases [5β-reductase and 5α-reductase]) and kidneys (through 11β-hydroxysteroid dehydrogenase type 2 [11β-HSD2] which converts cortisol to cortisone). This removal is offset by the regeneration of cortisol from cortisone through 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in liver and adipose tissue.[11,12] The regulation of these enzymes is complex.[12,13] In addition, in critically ill patients, elevated circulating levels of bile acids could be powerful suppressors of the expression and activity of cortisol metabolizing enzymes.[14-17]

We hypothesized that cortisol metabolism is reduced during critical illness, contributing to sustained hypercortisolemia with enhanced negative-feedback inhibition of ACTH.

3.3 MATERIALS & METHODS

To test our hypothesis, we performed five clinical studies comparing 158 consecutively screened patients in ICU with 64 demographically matched controls (Table 11 and Table-S1 in the Supplementary Appendix).[18,19] In these studies, we measured daily levels of ACTH and cortisol; plasma cortisol clearance, metabolism and production during infusion of deuterium-labeled steroid hormones as tracers; plasma clearance of 100 mg hydrocortisone; urinary cortisol metabolites and levels of messenger RNA (mRNA) and protein in liver and adipose tissue to assess major cortisol-metabolizing enzymes. Excluded from the study were patients and controls who had predisposing risk factors for HPA-axis dysfunction, who were receiving contraindicated drugs or who were undergoing extracorporeal membrane oxygenation or therapy with a circulatory-assist device (for details, see Supplementary Appendix, Methods S1-S2; Table-S2). All samples were stored at -80°C.

All study protocols and written consent forms were approved by the Institutional Ethical Review Board of the KU Leuven (ML1094/ML2707, ML1820/ML2707, ML4190/ML2112, ML6625, ML5754). All participants or their representatives provided written informed consent.

Plasma ACTH and cortisol time course

Morning blood was collected daily from 47 patients for 7 days after admission and from 12 controls (Table 1). Samples were collected in pre-chilled EDTA tubes, placed on ice and centrifuged at 4°C. Total levels of cortisol (Immunotech, Prague, Czech Republic) and CBG (DiaSource, Louvain-la-Neuve, Belgium) were quantified on radioimmunoassay and ACTH levels on double-monoclonal immunoradiometric assay (Brahms Diagnostics, Berlin, Germany).[20,21]

Plasma cortisol clearance and production

A total of 11 patients and 9 controls (Table 1) received intravenous deuterated cortisol ([9,11,12,12-$^2H_4$]-cortisol, D4-cortisol, Cambridge Isotopes)[22] as a 0.7 mg priming bolus followed by continuous infusion of 0.35 mg/h for 3 hours between 10 a.m. and 1 p.m. to achieve steady state in the circulation.[11,23,24] After 100 minutes of D4-cortisol infusion, participants also received deuterated cortisone ([1,2-$^2H_2$]-cortisone, D2-cortisone, Cambridge Isotopes)[25] as a 0.08 mg priming bolus, followed by continuous infusion of 0.1053 mg/h. Blood samples were obtained 5 minutes before D4–cortisol infusion and at intervals of 60, 120, 140, 160, 165, 170, 175 and 180 minutes after infusion.

Table 1: Characteristics of study patients and controls

	Plasma ACTH-Cortisol Time Course Study		D4-Cortisol Tracer Study		Plasma Clearance of a Therapeutic Dose of Cortisol Study		Cortisol Metabolizing Enzymes Urine Study		Cortisol Metabolizing Enzymes Tissue Biopsy Study	
	Patients (n=47)	**Controls (n=12)**	**Patients (n=11)**	**Controls (n=9)**	**Patients (n=20)**	**Controls (n=8)**	**Patients (n=36)**	**Controls (n=15)**	**Patients (n=44)**	**Controls (n=20)**
Demography and anthropometry										
Gender (no. male (%))	27 (57)	5 (42)	7 (64)	5 (56)	8 (40)	5 (63)	25 (69)	9 (60)	28 (63)	14 (70)
Age (yr) (mean±SD)	63.7 ± 18.0	60.4 ± 4.7	68.5 ± 8.3	62.6 ± 4.1	64.4 ± 13.2	60.3 ± 4.3	66.4 ± 10.8	61.1 ± 8.3	71.2 ± 12.0	70.4 ± 11.6
BMI (kg/m^2) (mean±SD)	26.2 ± 4.2	24.4 ± 3.4	28.5 ± 4.9	25.2 ± 4.1	26.3 ± 5.8	24.1 ± 1.8	26.7 ± 5.4	25.2 ± 5.3	24.8 ± 3.6	25.0 ± 2.6
Admission severity of illness score										
APACHE II (mean±SD)	28 ± 10		29 ± 11		34 ± 7		31 ± 6		29 ± 9	
Patients characteristics at study time										
SIRS (no. (%))	20 (43)		7 (64)		20 (100)		22 (61)		35 (80)	
Sepsis (no. (%))	16 (34)		5 (45)		15 (75)		17 (47)		26 (59)	
CRP (mg/l) (mean±SD)	107 ± 86		199 ± 131		143 ± 106		109 ± 81		185 ± 91	
Requiring Inotropes (no. (%))	6 (13)		3 (27)		8 (40)		2 (7)		24 (55)	
Requiring Vasopressors (no. (%))	17 (36)		7 (64)		20 (100)		19 (53)		30 (68)	
24h Urine Output (liter) (mean±SD)	1.8 ± 1.0		1.6 ± 0.5		0.9 ± 0.7		1.7 ± 0.7		1.0 ± 1.3	
Blood Lactate (mmol/l) (mean±SD)	0.93 ± 0.28		1.18 ± 0.35		2.54 ± 2.84		1.27 ± 1.85		3.50 ± 1.16	
Treated with Opioids (no. (%))	28 (60)		10 (91)		15 (75)		16 (44)		34 (77)	
On Therapeutic Anticoagulation (no. (%))	5 (11)		0 (0)		0 (0)		0 (0)		2 (5)	
Clinical Outcomes										
Days in ICU (mean±SD)	18 ± 16		26 ± 16		24 ± 32		22 ± 25		14 ± 17	
Day of Sampling (mean±SD)	1 to 7		7 ± 5		9 ± 15		10 ± 17		14 ± 17	
ICU Mortality (no. (%))	6 (13)		2 (18)		7 (35)		5 (14)		44 (100)	

The body-mass index (BMI) is the weight in kilograms divided by the square of the height in meters. Acute Physiology and Chronic Health Evaluation II (APACHE II) ranges from 0 to 71, with higher scores indicating greater severity of illness.[18] CRP: C-Reactive Protein, CO: Cardiac Output. SIRS: Systemic Inflammatory Response Syndrome, determined by the BONE criteria.[19]

In pre-infusion samples, plasma levels of ACTH (as described above) and levels of tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) were measured by means of enzyme-linked immunosorbent assay (ELISA) (Invitrogen, Camarillo, USA). Plasma concentrations or tracer/tracee ratios of endogenous cortisol and cortisone, 9,11,12,12-$[^2H]_4$-cortisol (D4-cortisol), [9,12,12-$^2H_3$]-cortisol (D3-cortisol), and 1,2-$[^2H]_2$-cortisone (D2-cortisone) were quantified by Liquid-Chromatography-tandem-Mass-Spectrometry (LC-MS/MS), with modification to the technique previously described.[11,25] Ten patients underwent a short ACTH-stimulation test (injection of 250 µg Synacthen®) within 24 hours after tracer infusion.

Cortisol kinetics were calculated from mean values in steady-state (t = +160 to +180 min). D4-cortisol clearance was calculated by dividing the D4-cortisol infusion rate by the steady-state concentration. Cortisol rate of appearance, a composite of adrenal cortisol secretion and extra-adrenal cortisol regeneration, was calculated as the D4-cortisol infusion rate divided by the D4-cortisol/cortisol ratio. Cortisol regeneration by 11β-HSD1 was estimated by the rate of appearance of D3-cortisol, calculated as the D4-cortisol infusion rate divided by the D4-cortisol/D3-cortisol ratio. Cortisone kinetics were also calculated from mean values in steady-state (t = +160 to +180 min). Net cortisone rate of appearance was calculated after adjustment for differences in substrate cortisol concentration as D2-cortisone infusion rate divided by [D2-cortisone/cortisone ratio]x[plasma cortisol]. The calculated rate of appearance is 'net' because the tracer D2-cortisone is converted to D2-cortisol by 11β-HSD1 reductase, and then recycled back to D2-cortisone by 11β-HSD2 dehydrogenase; similar recycling occurs between endogenous cortisol and cortisone. As a result, the dilution of D2-cortisone by endogenous cortisone underestimates the total 11β-HSD2 dehydrogenase activity by an amount that is proportional to this 'recycling' of tracer. However, when comparing subjects in whom there is no difference in 11β-HSD1 reductase activity (quantified by D3-cortisol generation, as above) then the extent of recycling of D2-cortisone tracer can be assumed not to be different, and any differences in net rate of appearance of cortisone can be attributed to 11β-HSD2 dehydrogenase. These circumstances applied in our comparison of ICU patients and controls.

Plasma clearance of a therapeutic dose of cortisol

A total of 20 patients and 8 controls (Table 1) received a 100 mg intravenous bolus hydrocortisone (Solu-Cortef®). Blood samples were taken every 10 minutes for 1 hour and then every hour for 4 hours. Cortisol was quantified by radioimmunoassay, as described above. Plasma clearance of cortisol was estimated by dividing the hydrocortisone dose by the area-under-the-curve of the plasma cortisol concentrations over time (estimated from 0 to infinity) and cortisol distribution volume was estimated by

dividing the calculated clearance by the elimination rate constant.[26,27] Plasma half-life, a composite measure of clearance and distribution volume, was estimated by dividing ln2 by the elimination rate constant, calculated from the slope of the regression line of the log-transformed cortisol concentration over time.[27]

Activity of cortisol metabolizing enzymes

We collected 24-hour urine samples from 36 patients and 15 controls, followed by a morning blood sample (Table 1). Patients requiring renal replacement therapy were excluded. We used LC-MS/MS to estimate levels of urinary Cortisol (F), cortisone (E), 5α-tetrahydrocortisol (allo-THF), 5β-tetrahydrocortisol (THF) and tetrahydrocortisone (THE), which were subsequently quantified on Gas-Chromatography-Mass-Spectrometry (GC-MS).[28] α-Reductase activity was estimated by the allo-THF/F ratio and 5β-reductase activity by THF/F and THE/E ratios. The activity of renal 11β-HSD2 was estimated by the E/F ratio and the overall activity of 11β-HSDs by (allo-THF+THF)/THE ratio, reflecting the balance of cortisol/cortisone interconversion.[28-32] In addition, total bile acids in the serum samples were quantified by means of enzymatic cycling (Diazyme, Poway, California).

Tissue Expression of Cortisol Metabolizing Enzymes

For this study, we investigated liver and adipose tissue biopsies collected immediately after death from 44 non-surviving patients (Table 1) who had not been treated with exogenous glucocorticoids during ICU stay.[33,34] The postmortem biopsy samples of liver (lower right quadrant) and abdominal subcutaneous and omental adipose tissue were harvested within minutes after death [adipose tissue 16 (10-24) min; liver 25 (17-40) min]. Blood samples were collected in the morning on the day of death. For comparison, liver and adipose tissue biopsies and blood samples were collected from 20 matched control patients undergoing elective abdominal surgery for restorative rectal resection. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until analysis. Blood samples were centrifuged and frozen at -20°C and stored at -80°C.

Total mRNA was purified from liver and adipose tissue using Qiazol lysis reagent (Qiagen, Hilden, Germany) and subsequently purified with RNAeasy Mini columns (Qiagen). Samples were treated with DNase to remove all genomic DNA, and 0.5 µg total mRNA was reverse-transcribed using random hexamers. Copy DNA (cDNA) levels of 11β-HSD1, 5α-reductase type-1 and 5β-reductase were quantified in real time with the TaqMan® gene expression assays using the StepOnePlus™ System (Applied Biosystems, Carlsbad, CA), for highly accurate quantification of mRNA levels. Individual samples with a copy number coefficient of variation greater than 20% were reanalyzed. Data are

expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and as a fold difference from the mean of the control patients. Primers and probe for 5α-reductase type-1 detection were developed using Primer Express® software (Applied Biosystems) and generated by Eurogentec (Liege, Belgium): forward, 5'-GGAATCTGTCTAGGAGCCCTCTCT-3'; reverse, 5'-TCCCCAAGTTCTCCACTTACACA-3'; probe, 5'-AAACTTGCCAACCTTCGTGTCAGGTGCT-3'. For 11β-HSD1, 5β-reductase and GAPDH, assays from Applied Biosystems were used (Hs01547870_m1, Hs00818881_m1 and 4310884E).

To quantify protein expression, liver samples were weighed and homogenized in lysis buffer with phosphatase and protease inhibitors, using ceramic beads and a Precellys 24 homogenizer (6000 rpm, 45 sec, Bertin Technologies, Villeurbanne, France). Protein content was determined using Coomassie Protein Assay Reagent (Pierce Biotechnology Inc., Rockford,IL). An equal amount of protein was loaded for each sample and homogenates were separated by denaturating SDS-PAGE and immunoblotted with specific antibodies against 5β-reductase (ab89166, Abcam, Cambridge, UK), against liver 11β-HSD1 (pc544, The Binding Site, Birmingham, UK), against adipose tissue 11β-HSD1 (ab39364, Abcam, Cambridge, UK), against 5α-reductase (sc20658, Santa Cruz Biotechnology, Heidelberg, Germany) and with species-specific HRP-conjugated secondary antibodies (P0447, P0448 and P0163, Dako, Heverlee, Belgium). Signals were quantified using ImageQuant™ software, and normalized for cytokeratin (CK)-18 protein expression (liver) and GAPDH protein expression (adipose tissue) and as a fold change of the mean of the control patients. The CK-18 antibody was purchased from Abcam (ab668, Cambridge, UK) and the GAPDH antibody from Ambion (AM4300, Austin, USA). Enzyme activity of 11β-HSD1 and 5β-reductase was determined *in vitro* in hepatic tissue, and enzyme activity of 11β-HSD1 was determined in adipose tissue.[35] Total cortisol was quantified with the use of chemiluminescence (Immulite®2000-Immunoassay; Diagnostic Products Corporation, Los Angeles, CA) and total bile acids were measured as described above.

Statistical Analyses

All data are presented as means ± standard deviation (SD) or medians (interquartile ranges(IQR)). We used Wilcoxon rank-sum test for data that did not have a normal distribution and unpaired Student's t-tests for normally distributed data. Proportions were compared with the use of chi-square tests. Associations were analyzed with linear regression after transformation to normal distribution when required. We used analysis of variance to calculate the significance of Pearson's determination coefficients (r^2). A two-sided P value of less than 0.05 was considered to indicate statistical significance. All statistical analyses were performed with JMP software, version 9.0.0 (SAS Institute Inc., Cary, USA).

3.4 RESULTS

Differences between patients and controls applied to survivors and non-survivors and were not influenced by illness severity, illness duration at the time of blood or tissue sampling, or status with respect to the use of opioids or anticoagulant agents (Tables S2 trough S6 in the Supplementary Appendix).

Plasma ACTH and cortisol time course

In the presence of elevated total cortisol levels (averaged over 7 days, P=0.01) patients had lower ACTH levels than did controls (P<0.001) (Figure 1). Levels of cortisol did not correlate with ACTH levels.

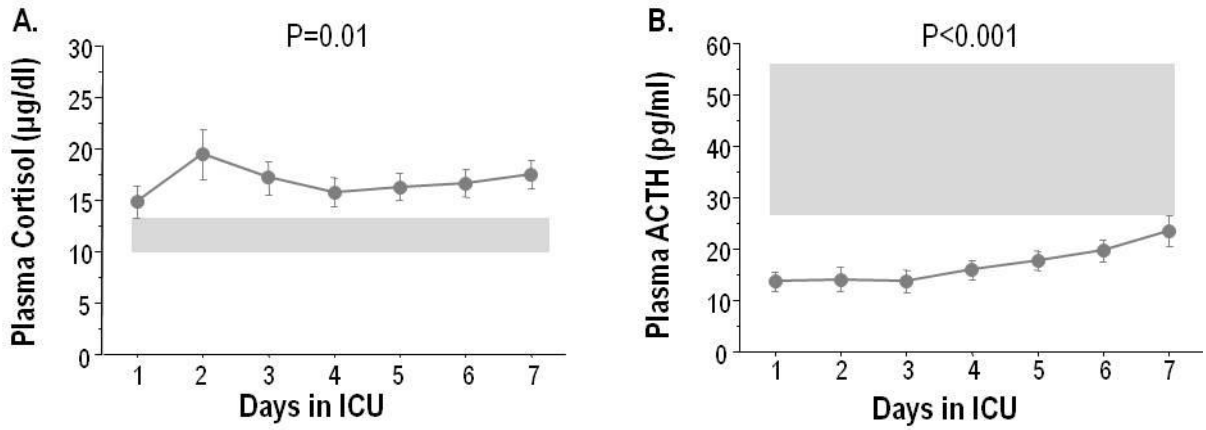

Figure 1- Plasma ACTH and Cortisol Time Course
Means and standard errors for ACTH and cortisol from day 1 to 7 in ICU are depicted for patients (dark grey) (N=47). The light grey area represents the interquartile range of values in healthy controls (N=12). The overall mean cortisol over the 7 days was 16.8±7.8 µg/dl for patients and 11.9±2.3 µg/dl for controls (P=0.01). The overall mean ACTH over the 7 days was 16.9±9.5 pg/ml for patients and 49.6±37.9 pg/ml for controls (P<0.001). For conversion of ACTH to SI units (pmol/l) multiply by 0.22. For conversion of cortisol to SI units (nmol/l) multiply by 27.6.

Mean levels of CBG were lower in the patients than in the controls with values for patients on day 1 of 31.6±10.4 mg/l (P=0.001) and on day 7 of 47.4±11.6 mg/l (P<0.001), as compared with a single measure of 67.8±8.7 mg/l in the controls. As a consequence, calculated median free cortisol levels were higher in the patients than in the controls with values for patients on day 1 of 1.0 µg/dl (IQR 0.4-2.7) and on day 7 of 0.9 µg/dl (0.6-1.6), as compared with a single measure of 0.4 µg/dl (0.3-0.4) in the controls (P≤0.001 for both comparisons).

Plasma cortisol clearance and production

Steady-state concentrations and enrichments of D4-cortisol and D2-cortisone were achieved from 60 minutes onward. In the patients, as compared with the controls, endogenous cortisol levels were increased by a factor 3.5 (P<0.001), and the rate of appearance of cortisol (hereafter referred to as cortisol production) was increased by 83% (P=0.02), but pre-infusion ACTH levels were reduced by 65% (P=0.03) (Figure 2 A,B,C).

There was no significant difference in cortisol production between patients who were treated with catecholamines and those who were not treated with catecholamines (2.7±1.3 mg/h and. 2.9±1.4 mg/h, respectively; P=0.86).

In the patients, as compared with the controls, plasma levels of TNF-α were increased by 49% (P=0.001) and plasma IL-6 were increased by more than a factor of 200 (P<0.001). Cytokine levels correlated positively with cortisol production (R^2=0.26 and P=0.02 for TNF-α and R^2= 0.30 and P=0.01 for IL-6); there also was a positive correlation after correction for ACTH levels, (R^2=0.28 and P=0.03 for TNF-α and R^2= 0.30 and P=0.02 for IL-6). Consistent with these correlations was the finding that patients with the systemic inflammatory response syndrome (SIRS)[19] had 90% higher cortisol production than those who did not have the syndrome (3.4±1.1 mg/h vs. 1.8±1.1 mg/h, P=0.04); the latter not differ significantly from that of controls (P=0.39).

In the patients, as compared with the controls, D4-cortisol levels during infusion were 57% higher (P=0.04) and plasma clearance of D4-cortisol was reduced by 53% (P=0.03) (Figure 2 D,E). Reduced cortisol clearance did not correlate with markers of organ perfusion (Table S7 in the Supplementary Appendix).

We tested whether decreased cortisol clearance was associated with evidence of adrenal insufficiency in relation to reduced ACTH stimulation. A lower cortisol response to ACTH stimulation in the patients correlated both with lower cortisol production and with lower D4-cortisol clearance (Figure 2 F,G). Patients with a cortisol response to ACTH <21μg/dl, a level that is considered indicative of absolute adrenal insufficiency,[36] had substantially lower D4-cortisol clearance (0.15±0.02 l/min) than did patients who had a normal response to ACTH (0.28±0.11 l/min; P=0.01). Cortisol production in patients with adrenal insufficiency (1.4±0.5 mg/h) was indistinguishable from that in controls, whereas it was elevated (3.0±1.3 mg/h) in patients with a normal response to ACTH (P=0.03). Circulating levels of cortisol before ACTH stimulation were similar in these two groups of patients (9.5±1.5μg/dl and 11.5±6.5 μg/dl, respectively; P=0.51).

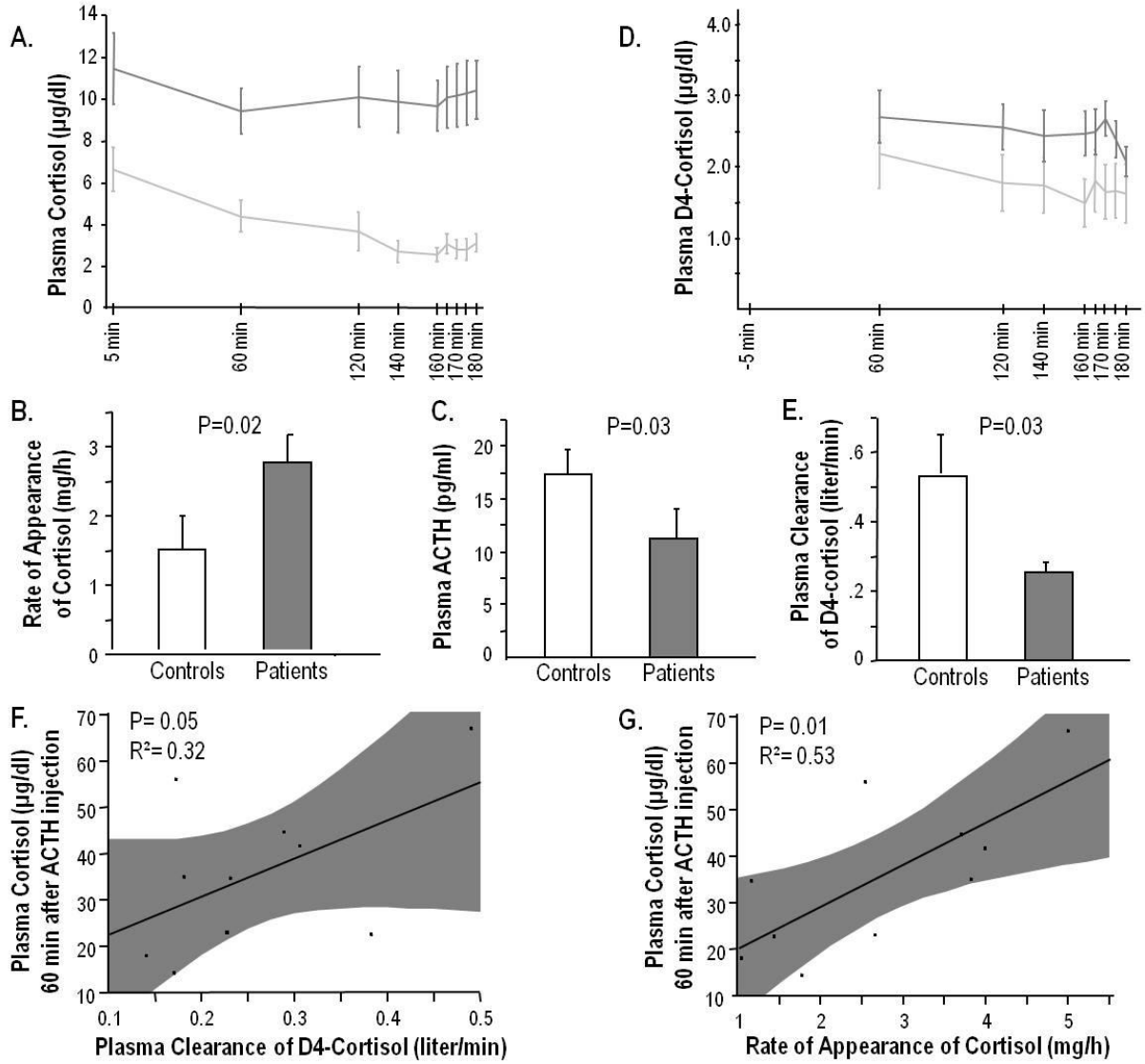

Figure 2 - Results of D4-cortisol tracer infusion

Panel A and D: Plasma levels of endogenous cortisol and of D4-cortisol tracer reached steady state during study period in patients (N=11) and controls (N=9). Means and standard errors are depicted for patients (dark grey) and controls (white/light grey). For conversion of cortisol to SI units (nmol/l) multiply by 27.6. For conversion of D4-cortisol to SI units (nmol/l) multiply by 27.3. Panel B depicts Rate of Appearance of Cortisol. Panel C depicts pre-infusion plasma ACTH levels. For conversion of ACTH to SI units (pmol/l) multiply by 0.22. Panel E depicts D4-clearance. Bar charts in panels B, C and E represent means and standard errors. Panels F-G show the correlation between Plasma Clearance of D4-cortisol and Rate of Appearance of Cortisol with plasma cortisol responses, 60 minutes after ACTH injection (250 µg) amongst patients. The shaded area represents 95% confidence interval.

Tracer analysis also allowed dissection of the contribution of 11β-HSD enzymes to altered cortisol clearance.[11,25] The patients had a lower net rate of appearance of cortisone than the controls (0.07±0.02 mg/h for every µg/dl vs. 0.14±0.07 mg/h for every µg/dl, P=0.01). However, there was no significant between-group difference in the level of regeneration of cortisol by 11β-HSD1, as measured by the rate of appearance of D3-cortisol (0.42±0.12 mg/h and 0.49±0.12 mg/h, respectively; P=0.23). These findings are consistent with impaired conversion of cortisol to cortisone by 11β-HSD2.

Plasma clearance of a therapeutic dose of cortisol

We tested whether altered cortisol metabolism in critically ill patients occurs at supra-physiological concentrations after the administration of therapeutic hydrocortisone. The calculated plasma clearance after the administration of 100 mg of hydrocortisone in the patients was 60% lower than that in the controls, with a distribution volume that was 37% higher (Figure 3). Cortisol clearance was even more suppressed in non-survivors (0.02±0.01l/min) than in survivors (0.05±0.03 l/min; P=0.03). The plasma half-life of the 100 mg bolus was a mean 7-fold longer in patients than controls (Figure 3), even more pronounced in non-survivors [9.4 (8.9-31.2) hour] than in survivors [5.6 (3.1-7.5) hour] (P=0.03).

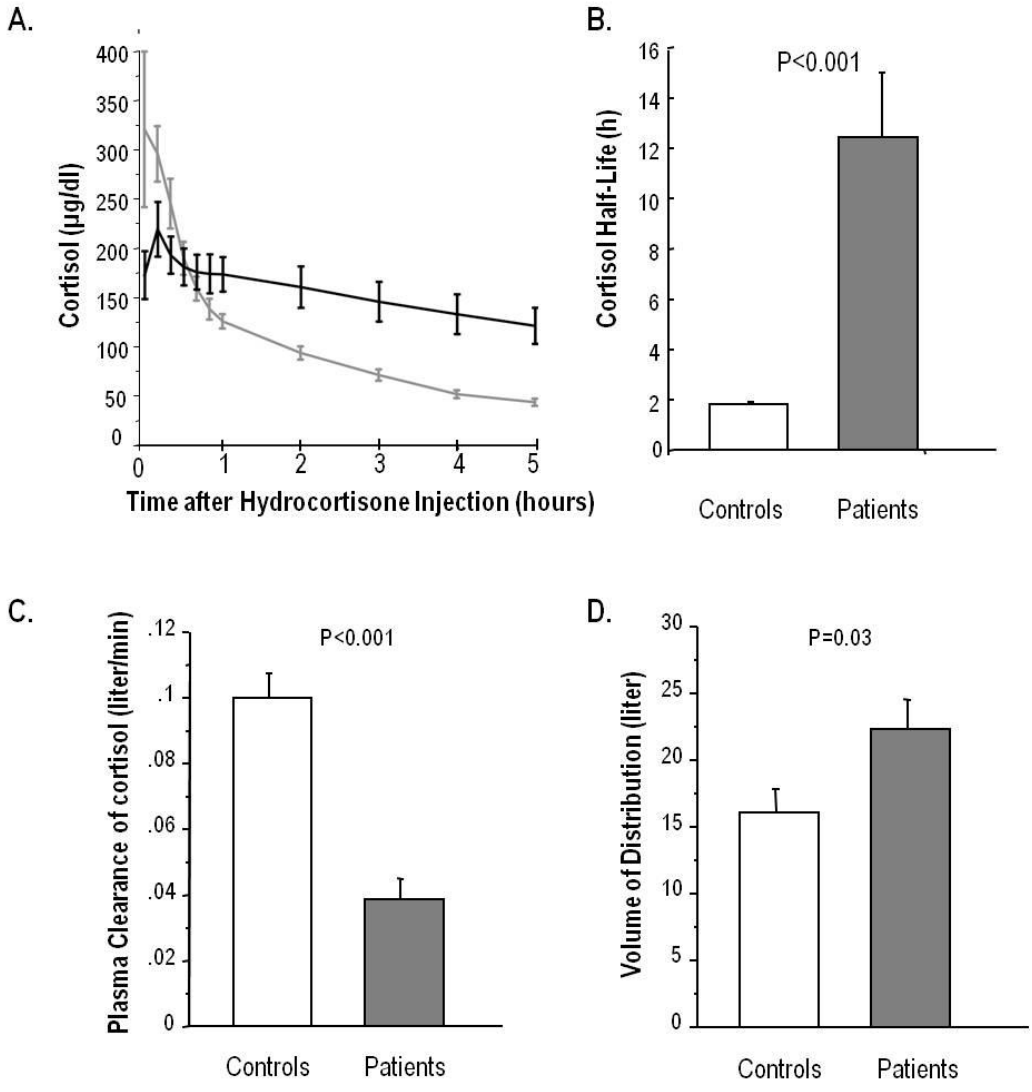

Figure 3 - Plasma Clearance of a Therapeutic Dose of Cortisol
Cortisol half-life (hour) was determined in 20 patients and 8 controls. Panel A depicts the means and standard errors of the plasma cortisol concentration time course after injection of 100 mg hydrocortisone in patients (dark grey lines) and controls (light grey lines). Panel B depicts the half-life. For conversion of cortisol to SI units (nmol/l) multiply by 27.6. Panel C and D depict the plasma clearance and the volume of distribution, respectively, of a 100 mg dose of hydrocortisone. Bar charts in panels B-D represent means and standard errors.

Activity of cortisol metabolizing enzymes

We addressed the contribution of 11β-HSDs enzymes and A-ring reductases to impaired cortisol clearance in patients. There were no correlations of urinary levels of cortisol metabolites and creatinine clearance or daily urine output, which was similar in patients (1936±655 ml/24h) and controls (1713±734 ml/24h) (P=0.29).[37,38] LC-MS/MS analysis suggested substantial changes in relative excretion of cortisol metabolites (Table S8 in Supplementary Appendix). These findings were further quantified on GC-MS, which showed that the daily urinary excretion of cortisol was increased by a factor of 3.2 in the patients, as compared with the controls, and urinary excretion of cortisone was 73% higher in the patients (P<0.001 for both comparisons). In contrast, levels of allo-THF and THF were similar in patients and controls, whereas the levels of THE were reduced by 69% in the patients (P<0.001) (Table S9 in Supplementary Appendix). Decreased levels of 11β-HSD2 were confirmed in the patients, in whom the ratio of urinary E/F, which reflects the renal 11β-HSD2 level and the ratio (THF+allo-THF)/THE, which reflects the conversion of cortisol to cortisone, was markedly altered in favor of cortisol (Figure 3A and 3B). In addition, the ratios reflecting activities of A-ring reductases were markedly reduced in patients, as compared with the controls (Figure 4 C,D,E). Non-survivors had lower estimated 5α-reductase activity than survivors (0.6 [0.2-0.8] versus 1.4 [0.8-4.2]; P=0.01) but similar 5β-reductase activity (5β-THF/F 1.2 [0.2-2.5] vs. 1.9 [1.3-3.3]; P=0.14). Levels of total bile acids were increased by a factor of 2.4 in the patients, as compared with the controls. (10.9±2.5 μmol/l versus 4.6±1.8 μmol/l; P=0.04) and correlated inversely with urinary ratios reflecting A-ring reductase activities (Figure 4 F,G,H).

Tissue Expression of Cortisol Metabolizing Enzymes

To test the inferences from urinary metabolite ratios and tracer kinetics, we studied cortisol metabolizing enzymes in tissue biopsy samples. Circulating levels of total cortisol in the patients were three times as high as those in the controls (24.3 μg/dl [19.6-30.3] versus 7.4 μg/dl [4.9-14.7]; P<0.001). Levels of total bile acids were substantially higher in the patients than in the controls (P<0.001) (Figure 5A) and correlated positively with cortisol (R^2=0.26; P<0.001).

In liver specimen from the patients, the mRNA and protein expression of 5β-reductase was reduced by 80 to 91% as compared with controls (Figure 5 B,C), and enzyme activity was reduced by 58% (0.022 pmol/min/mg [0.012-0.029] versus 0.053 pmol/min/mg [0.033-0.067] (P=0.01). The mRNA levels correlated positively with protein expression of 5β-reductase (R^2=0.46; P<0.001), which in turn had a negative correlation with circulating cortisol levels (Figure 5 D). The level of total bile acids correlated inversely with the mRNA and protein expression of 5β-reductase (Figure 5 E;F).

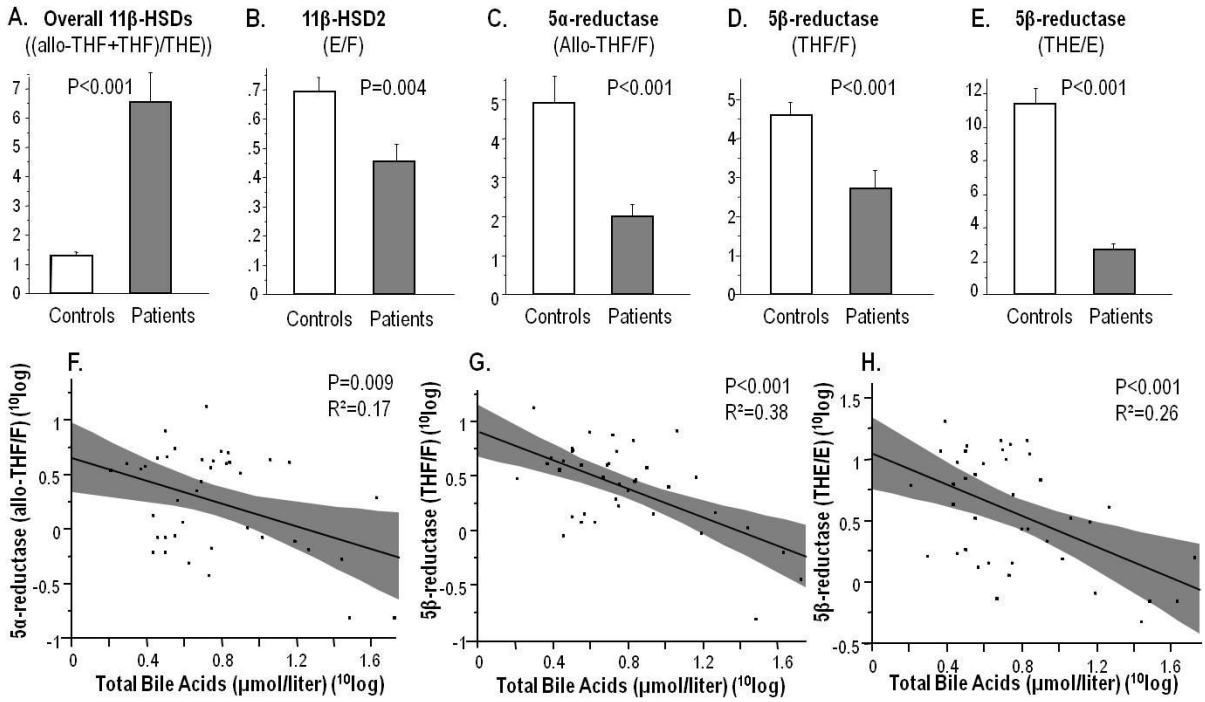

Figure 4 - Activity of cortisol-metabolizing enzymes estimated from ratios of cortisol metabolites in 24h-urine *Enzyme activities were estimated for 36 patients and 15 controls based on urinary metabolites quantified using GC-MS. 5α-Reductase activity was estimated by the allo-THF/F ratio and 5β-reductase activity by THF/F and THE/E ratios. The activity of renal 11β-HSD2 was estimated by the E/F ratio and the overall activity of 11β-HSDs by (allo-THF+THF)/THE ratio, reflecting the balance of cortisol/cortisone interconversion. Bar charts represent means and standard errors. The shaded area represents 95% confidence interval.*

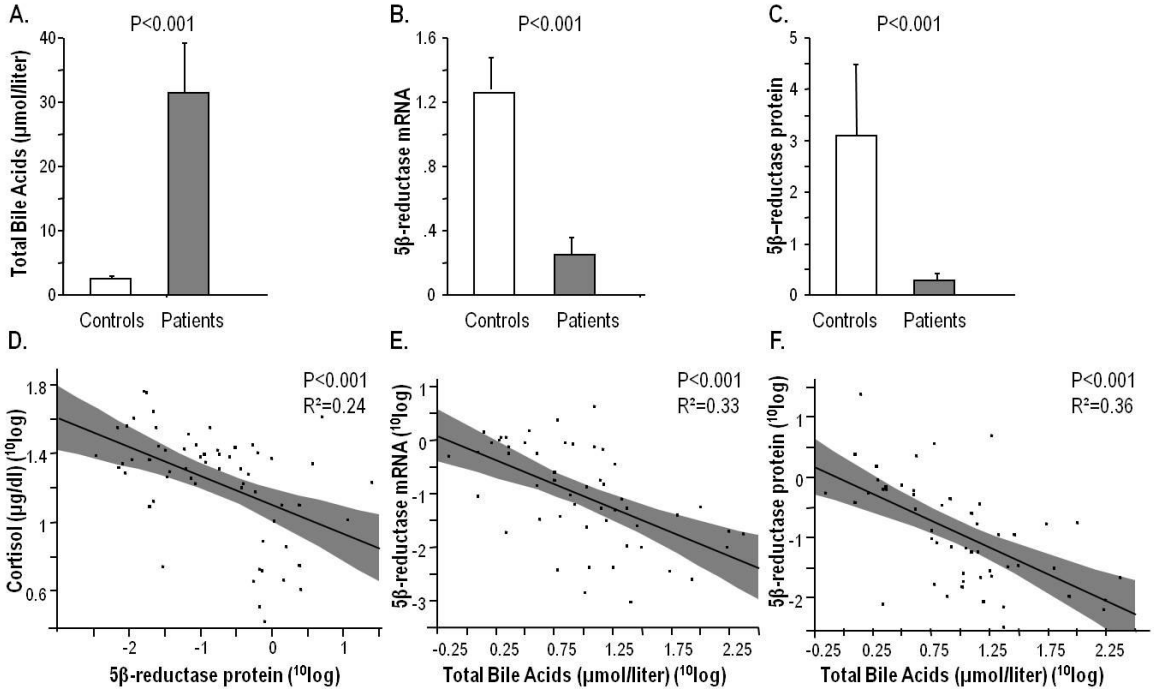

Figure 5 - Tissue expression of cortisol metabolizing enzymes in relation to circulating cortisol and bile acids. *Twenty controls and 44 patients were studied. Bar charts represent means and standard errors. For conversion of cortisol to SI units (nmol/l) multiply by 27.6. The mRNA data are expressed, normalized to GAPDH, as a fold difference from the mean of the controls. Protein data are expressed normalized for CK-18 protein expression, as a fold difference from the mean of the controls.*

The liver mRNA level of 5α-reductase was reduced by 77% in patients, as compared with controls (0.23 [0.12-0.36] vs. 1.01 [0.66-1.65]; P<0.001). The hepatic mRNA 5α-reductase level also correlated negatively (albeit more weakly than 5β-reductase) with circulating bile acids (R^2=0.22, P<0.001) and cortisol (R^2=0.13; P=0.005).

Levels of 11β-HSD1 mRNA in liver were reduced by 80% in patients as compared to controls (0.20 [0.12-0.37] vs.1.01 [0.62-1.36]; P<0.001) whereas 11β-HSD1 protein and enzyme activity were unaltered. These levels were unrelated to the elevated cortisol levels, despite an inverse correlation with bile acids (R^2=0.26; P<0.001 for mRNA and R^2=0.16; P=0.005 for protein).

Levels of 11β-HSD1 mRNA in omental adipose tissue were reduced by 73% in patients as compared with controls (0.27 [0.16-0.46] vs. 1.00 [0.60-2.09]; P=0.003) and by 82% in subcutaneous adipose tissue (0.18 [0.07-0.30] vs. 1.00 [0.56-2.17]; P<0.001). These levels were unrelated to elevated cortisol levels, despite an inverse correlation with bile acids (subcutaneous adipose tissue R^2=0.28; P=0.008). Levels of 11β-HSD1 protein and in vitro enzyme activity in adipose tissue were unaltered.

3.5 DISCUSSION

In our study, elevated cortisol levels in critically ill patients were only partially explained by an increase of 83% in cortisol production as compared with controls. Since ACTH levels were paradoxically low in the patients, a pituitary-independent mechanism was suggested. We showed that impaired cortisol clearance contributed to hypercortisolemia, as suggested by studies conducted in the 1950s before the advent of ICUs.[39,40] Reduced cortisol clearance could be explained by suppressed levels of A-ring reductases and 11β -HSD2.

In other circumstances of reduced cortisol metabolism, such as congenital 11β-HSD2 deficiency,[41] negative feedback on the HPA-axis results in compensatory downregulation of cortisol secretion, with lower ACTH levels and adrenocortical atrophy. Elevated levels and production of cortisol in patients treated in the ICU must reflect an ongoing stimulus to cortisol secretion. In the presence of low ACTH, increased ACTH sensitivity might play a role. However, this seems unlikely during critical illness as cortisol responses to ACTH stimulation are not increased. More likely candidates are neuropeptides, catecholamines or cytokines,[10] especially since cytokine levels were substantially elevated and were positively correlated with cortisol production. The role of cytokines is further corroborated by the finding that only patients with pronounced inflammation had levels of cortisol production that were higher than the levels in controls, whereas cortisol clearance was suppressed regardless of the inflammatory status. It remains to be investigated whether adrenocortical atrophy is associated with a sustained reduction in the activation of ACTH receptors on adrenocortical cells in patients with reduced cortisol clearance, predisposes to adrenocortical atrophy remains to be investigated. However, such a mechanism would explain the high incidence of adrenal vascular instability in surgical patients with prolonged critical illness[42] and is supported by our observation that the patients with the least response to ACTH stimulation had the lowest cortisol production and the lowest cortisol clearance, despite a similar baseline cortisol level as compared with the other patients.

Although in isolation each of the separate studies is suggestive, the corroboration of the findings with the use of multiple approaches is helpful for making conclusions. Urinary excretion of cortisol was elevated in the critically ill patients, but cortisol metabolites were normal or low, despite increased cortisol production; this pattern is quite different from that in Cushing's syndrome.[30,43] The ratios of urinary cortisol metabolites suggested reduced activity of the A-ring reductases in the critically ill patients and a net suppression of cortisol to cortisone conversion. This interpretation was corroborated by low mRNA and protein levels and low activity of the A-ring reductases in liver-biopsies samples. Unfortunately, kidney samples were unavailable to quantify 11β-HSD2. However, the stable isotope

study showed impaired cortisone generation in the critically ill patients, indicating suppressed 11β-HSD2 activity. Moreover, 11β-HSD1 protein and enzyme activity in biopsy samples and *in vivo* D3-cortisol generation were unaltered, so it is unlikely that altered regeneration of cortisol from cortisone played a role in the patients.

Although hypoperfusion of cortisol-metabolizing organs could theoretically reduce cortisol breakdown, this factor does not explain our findings. In contrast, bile acids are known to be competitive inhibitors and transcriptional suppressors of cortisol metabolizing enzymes.[14-16] Observations in patients and animals with cholestasis support the inhibition of glucocorticoid metabolism by bile acids.[14,15,44] The negative correlation between the expression and activity of the A-ring reductases and circulating bile acid levels suggested that elevated levels of bile acids may reduce cortisol metabolism in critically ill patients, a hypothesis that should be further investigated.

Our studies have some limitations. First, it would have been ideal to document all the changes in one single patient population; this was not feasible, in part due to ethical reasons. However, the five groups of patients were matched, and the results of all studies corroborated our hypothesis of reduced cortisol breakdown. Second, biopsy samples were obtained on autopsy, which may have introduced bias. However, reduced cortisol clearance was clearly also present in the patients who survived.

These findings have important clinical implications. The contribution of reduced cortisol breakdown to hypercortisolemia during critical illness changes our understanding of the stress response. Reduced inactivation of cortisol may be important not only to increase circulating levels but also to potentiate cortisol levels and activity within the vital tissues that express inactivating enzymes. More pragmatically, the data suggest that “stress-doses” of hydrocortisone (200 mg/day), which are advocated to replace cortisol production in critically ill patients who are presumed to suffer from adrenal failure, are at least three-fold too high.[21] Finally, our data suggest that a low cortisol response to ACTH stimulation does not necessarily reflect adrenal failure, since cortisol production in critically ill patients is not subnormal and the suppressed clearance maintains hypercortisolemia. Our results may therefore help to explain why studies investigating the effect of the daily administration of 200 mg hydrocortisone in patients with sepsis (on the basis of a low cortisol response to ACTH stimulation), have had conflicting results.[5,7]

In conclusion, in critically ill patients in the ICU, reduced cortisol breakdown appeared to contribute to abnormal blood cortisol in critical illness. This finding has potential implications for the diagnosis of adrenal failure and its treatment in the ICU setting.

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SUPPLEMENTARY APPENDIX

Methods S1. Details of selection of patients and controls

Healthy controls

For the *in vivo* studies, healthy men and women were recruited among the relatives and friends of the research team and selected based on BMI, age and gender, to match with the patient groups. They were invited to participate after screening for exclusion criteria and investigation of their medical history:

- Treatment with glucocorticoids, other steroids or anti-steroid chemotherapy by any route in the previous 3 months
- Recent treatment with azoles, phenytoin, rifampicin, glitazones, imipramine, barbiturates or phenothiazines
- Cerebral or pituitary disease
- Adrenal dysfunction (Cushing's syndrome or Addison's disease)

Before the start of the study each healthy volunteer was examined by a medical doctor to confirm their healthy psychological and physical condition. The study was started after written informed consent was given.

For the biopsy studies, the controls were 20 matched patients undergoing elective abdominal surgery for restorative rectal resection. These were also consecutive patients, gender and age-matched who were invited to participate after screening for exclusion criteria (such as described above) before they were approached for consent.

Patient selection

During the time frames of the different clinical studies, consecutive adult (age ≥ 18y) patients were screened for eligibility on a daily basis, from the population present in 5 ICUs (total of 73-beds) from 2 departments (medical and surgical) in 1 University Hospital (University Hospital of Leuven).

Exclusion criteria

- Predisposing factors of adrenal insufficiency
    - Cerebral disease with intracranial hypertension threatening the neuroendocrine system
    - Pituitary disorders including (pan)hypopituitarism
    - Known adrenal disease (Cushing's syndrome or Addison's disease)

- Treatment with glucocorticoids, other steroids or anti-steroid chemotherapy within the last 3 months
- Intubation with etomidate within the last 72 hours
- Other drugs predisposing to glucocorticoid insufficiency[160]*:* azoles, phenytoin, rifampicin, glitazones, imipramin, phenothiazine, phenobarbital
- Liver failure

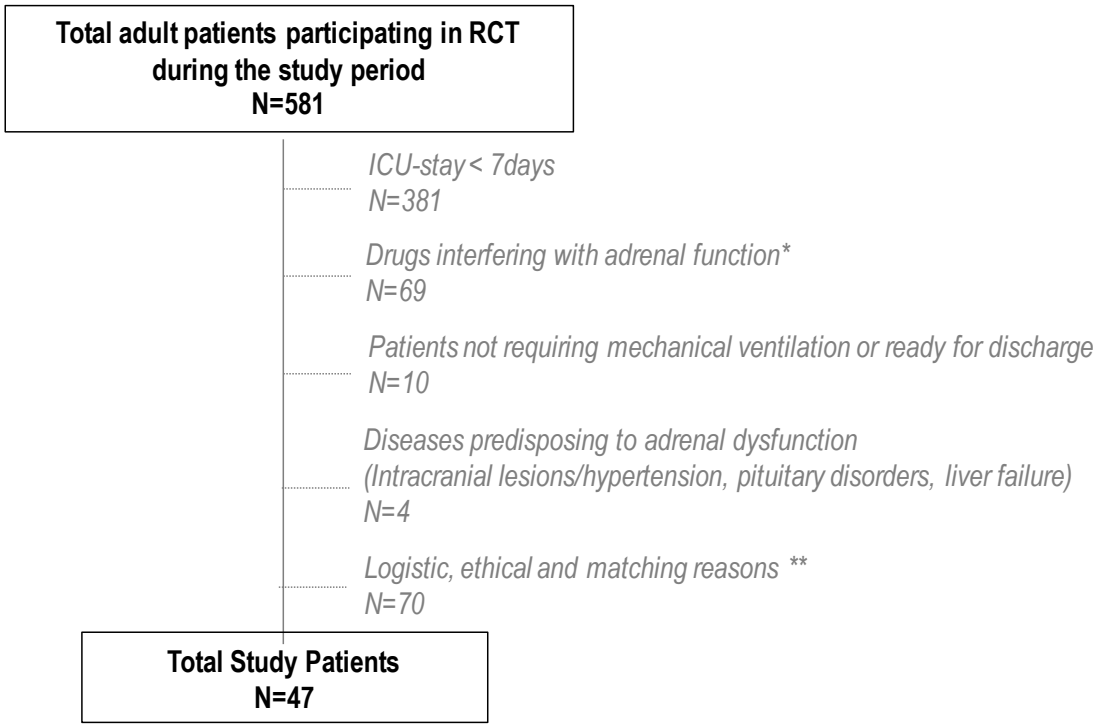
- Patients not critically ill enough
  - Patients not requiring mechanical ventilation
  - Patients expected to be discharged from ICU within the next 24 hours
- Patients on Extra-Corporeal Circulatory assist devices
- Patients with a planned intervention during the study period.
- No arterial line in place
- Ethical restrictions
  - Do not resuscitate (DNR) order
  - Patients expected to die within the next 24 hours
  - Declined participation

The patients were matched for demographical and illness-related characteristics with the previous studies. Inevitably the study on the clearance of a therapeutic dose of hydrocortisone was done in even more severely ill patients, those for whom clinicians considered it necessary to treat with hydrocortisone. Also the study of the biopsies was done in the sickest patients, as these were the non-survivors only.

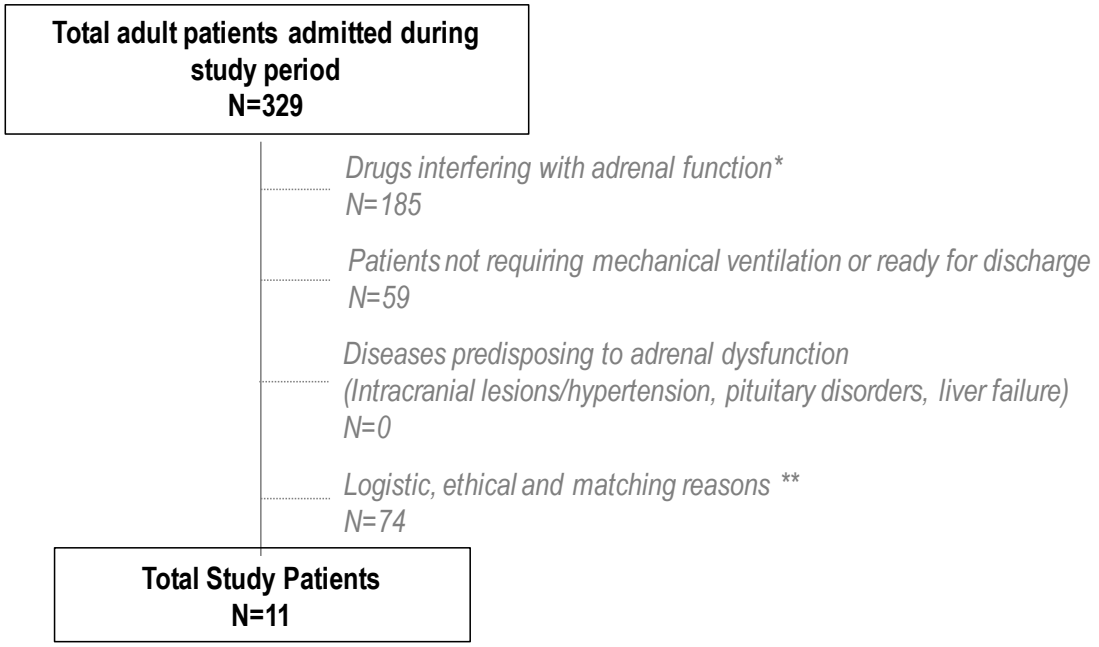
To exclude a biased selection of patients, we also analyzed the difference in the patient characteristics of those who were included in the study versus those not included and it is clear that, as intended, the studied patients do represent the severely ill patients in ICU. Demographical characteristics were comparable (Table S1).

Methods S2. Consort diagrams of all studies

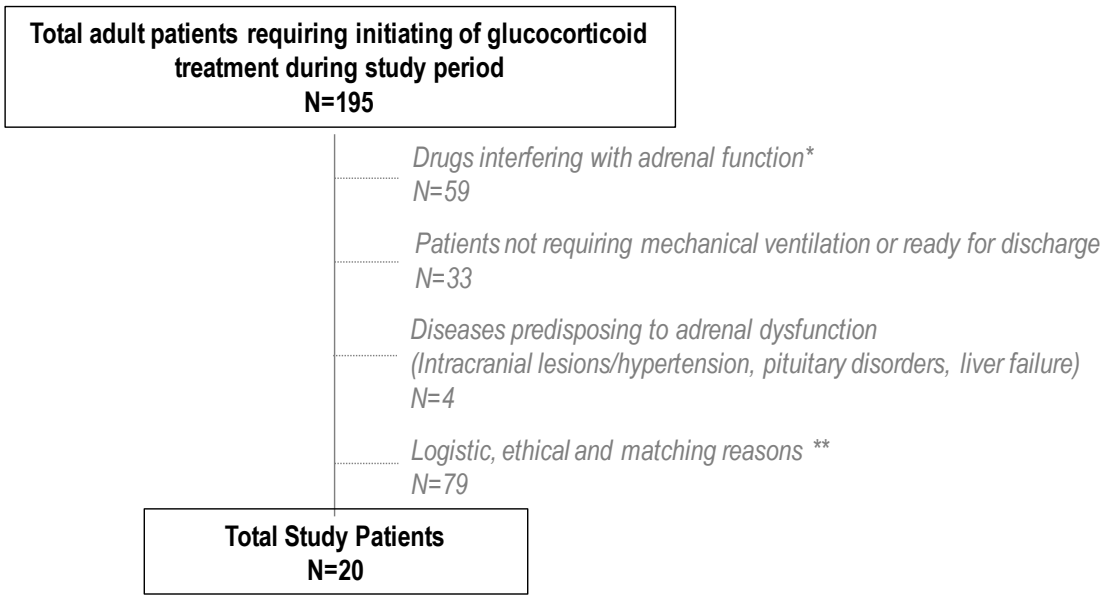
Study 1: Plasma ACTH-Cortisol Time Course Study



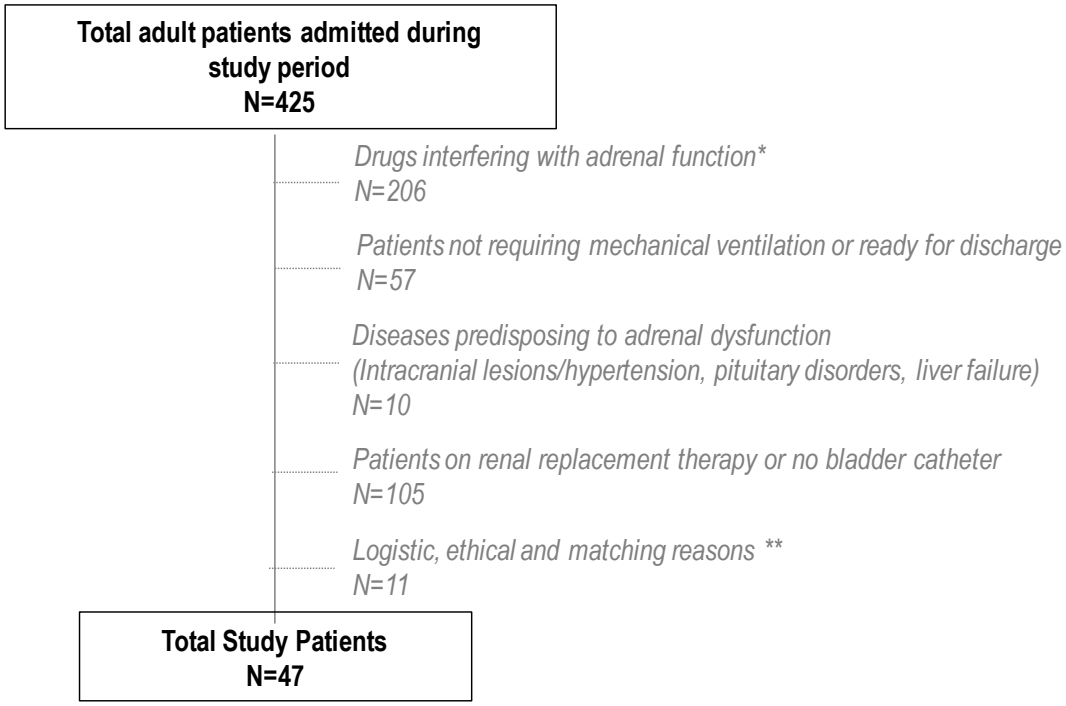
Study 2: D4-Cortisol Tracer Study



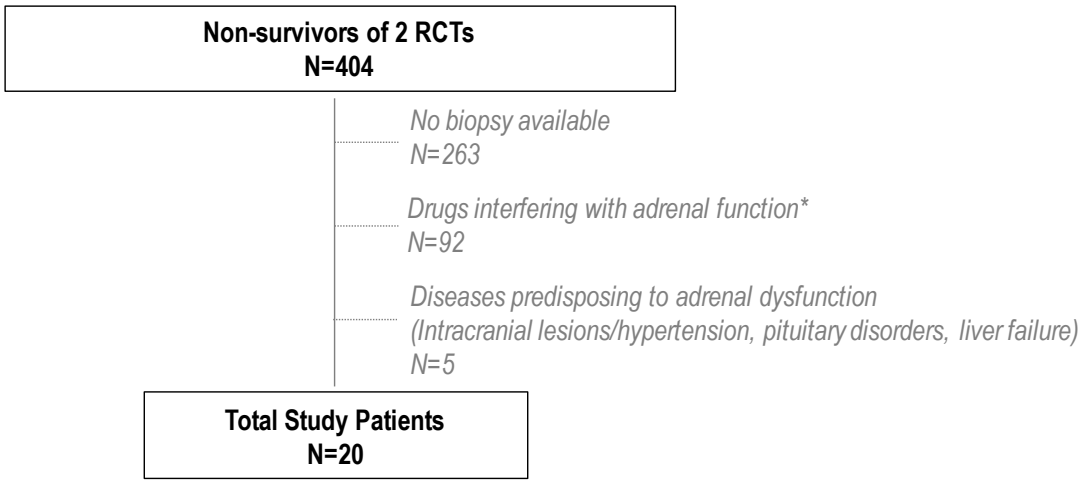
Study 3: Plasma Clearance of a Therapeutic Dose of Cortisol Study



Study 4 Cortisol Metabolizing Enzymes Urine Study



Study 5: Cortisol Metabolizing Enzymes Tissue Biopsy Study



** All types of glucocorticoids or anti-steroid chemotherapy, etomidate, azoles, phenobarbital, phenytoin, rifampicin, glitazones, imipramine, phenothiazine*

*** Planned surgical or diagnostic intervention, no arterial line, DNR code, circulatory assist devices, unavailability of the researchers, and matching with previous studies, declined participation*

(excluded in order of listing)

Table S1: Extended characteristics of study patients and controls

	Plasma ACTH-Cortisol Time Course Study		D4-Cortisol Tracer Study		Plasma Clearance of a Therapeutic Dose of Cortisol Study		Cortisol Metabolizing Enzymes Urine Study		Cortisol Metabolizing Enzymes Tissue Biopsy Study	
	Patients (n=47)	**Controls (n=12)**	**Patients (n=11)**	**Controls (n=9)**	**Patients (n=20)**	**Controls (n=8)**	**Patients (n=36)**	**Controls (n=15)**	**Patients (n=44)**	**Controls (n=20)**
Demography and anthropometry										
Gender (no. male (%))	27 (57)	5 (42)	7 (64)	5 (56)	8 (40)	5 (63)	25 (69)	9 (60)	28 (63)	14 (70)
Age (yr) (mean±SD)	63.7 ± 18.0	60.4 ± 4.7	68.5 ± 8.3	62.6 ± 4.1	64.4 ± 13.2	60.3 ± 4.3	66.4 ± 10.8	61.1 ± 8.3	71.2 ± 12.0	70.4 ± 11.6
BMI (kg/m^2) (mean±SD)	26.2 ± 4.2	24.4 ± 3.4	28.5 ± 4.9	25.2 ± 4.1	26.3 ± 5.8	24.1 ± 1.8	26.7 ± 5.4	25.2 ± 5.3	24.8 ± 3.6	25.0 ± 2.6
Admission characteristics										
APACHE II (mean±SD)	28 ± 10		29 ± 11		34 ± 7		31 ± 6		29 ± 9	
SIRS (no. (%))	43 (91)		10 (91)		20 (100)		36 (100)		35 (80)	
Sepsis (no. (%))	17 (36)		5 (45)		11 (55)		20 (56)		17 (39)	
Diagnostic group on admission (no. (%))										
Cardiovascular	22 (47)		4 (36)		8 (40)		7 (19)		16 (36)	
Respiratory/Esophageal-lung surgery	1 (2)		1 (9)		6 (30)		4 (11)		6 (14)	
Abdominal/Gastro-intestinal/Hepatic	6 (13)		2 (18)		4 (20)		13 (36)		7 (16)	
Other	18 (38)		4 (36)		2 (10)		12 (33)		15 (34)	
Patients characteristics at study time										
SIRS (no. (%))	20 (43)		7 (64)		20 (100)		22 (61)		35 (80)	
Sepsis (no. (%))	16 (34)		5 (45)		15 (75)		17 (47)		26 (59)	
CRP (mg/l) (mean±SD)	107 ± 86		199 ± 131		143 ± 106		109 ± 81		185 ± 91	
Last CO before Study (l/min) (mean±SD)	5.5 ± 1.2		5.4 ± 1.4		5.2 ± 1.5		6.9 ± 3.3		NA	
Requiring Inotropes (no. (%))	6 (13)		3 (27)		8 (40)		2 (7)		24 (55)	
Requiring Vasopressors (no. (%))	17 (36)		7 (64)		20 (100)		19 (53)		30 (68)	
24h Urine Output (liter) (mean±SD)	1.8 ± 1.0		1.6 ± 0.5		0.9 ± 0.7		1.7 ± 0.7		1.0 ± 1.3	

Blood Lactate (mmol/l) (mean±SD)	0.93 ± 0.28	1.18 ± 0.35	2.54 ± 2.84	1.27 ± 1.85	3.50 ± 1.16
Treated with Opioids (no. (%))	28 (60)	10 (91)	15 (75)	16 (44)	34 (77)
On Therapeutic Anticoagulation (no. (%))	5 (11)	0 (0)	0 (0)	0 (0)	2 (5)
Clinical Outcome					
Days in ICU (mean±SD)	18 ± 16	26 ± 16	24 ± 32	22 ± 25	14 ± 17
Day of Sampling (mean±SD)	1 to 7	7 ± 5	9 ± 15	10 ± 17	14 ± 17
ICU Mortality (no. (%))	6 (13)	2 (18)	7 (35)	5 (14)	44 (100)
Hospital Mortality (no. (%))	10 (21)	4 (36)	9 (45)	6 (17)	44 (100)

Table S2: Characteristics of patients selected and patients not selected for each study

	Plasma ACTH-Cortisol Time Course Study		D4-Cortisol Tracer Study		Plasma Clearance of a therapeutic dose of Cortisol study		Cortisol Metabolizing Enzymes Urine Study		Cortisol Metabolizing Enzymes Tissue Biopsy Study	
	Patients selected for study (n=47)	**Patients not selected for study (n=534)**	**Patients selected for study (n=11)**	**Patients not selected for study (n=318)**	**Patients selected for study (n=20)**	**Patients not selected for study (n=175)**	**Patients selected for study (n=36)**	**Patients not selected for study (n=389)**	**Patients selected for study (n=44)**	**Patients not selected for study (n=360)**
Demography and anthropometry										
Gender (no.(% male))	27 (57)	341 (64)	7 (64)	197 (62)	8 (40)	94 (54)	25 (69)	247 (64)	28 (63)	227 (63)
Age (yr) (mean ± SD)	63.7 ± 18.0	63.0 ± 14.1	68.5 ± 8.3	62.6 ± 16.0	64.4 ± 13.2	61.3 ± 15.2	66.4 ± 10.8	57.4 ±16.8	71.2 ± 12.0	64.5 ± 15.2
BMI (kg/m^2) (mean ± SD)	26.2 ± 4.2	26.4 ± 5.2	28.5 ± 4.9	25.3 ± 4.9	26.3 ± 5.8	26.4 ± 6.2	26.7 ± 5.4	26.4 ± 5.8	24.8 ± 3.6	24.6 ± 5.1
Admission characteristics										
APACHE II (mean ± SD)	28 ± 10	23 ± 11	29 ± 11	23 ± 9	34 ± 7	21 ± 9	31 ± 6	24 ± 10	29 ± 9	36 ± 13
SIRS (no. (%))	43 (91)	508 (95)	10 (91)	165 (52)	20 (100)	82 (47)	36 (100)	245 (57)	35 (80)	298 (83)
Sepsis (no. (%))	17 (36)	122 (23)	5 (45)	84 (26)	11 (55)	37 (21)	20 (56)	155 (36)	26 (59)	238 (66)
Diagnostic group on admission (no.)										
Cardiovascular	22 (47)	329 (62)	4 (36)	116 (36)	8 (40)	44 (25)	7 (19)	108 (28)	16 (36)	36 (10)
Respiratory/Esophageal -lung surgery	1 (2)	82 (15)	1 (9)	63(20)	6 (30)	38 (22)	4 (11)	80 (21)	6 (14)	145 (40)
Abdominal/Gastro-intestinal/Hepatic	6 (13)	55 (10)	2 (18)	57(18)	4 (20)	46 (26)	13 (36)	60 (15)	7 (16)	54 (15)
Other	18 (38)	68 (13)	4 (36)	82 (26)	2 (10)	47 (27)	12 (33)	141 (36)	15 (34)	125 (35)
Clinical Outcome										
Days in ICU (mean ± SD)	18 ± 16	8 ± 14	26 ± 16	12 ± 20	24 ± 32	11 ± 20	22 ± 25	32 ± 44	14 ± 17	16 ± 18
ICU mortality (no (%))	6 (13)	32 (6)	2 (18)	39 (12)	7 (35)	15 (9)	5 (14)	52 (13)	44 (100)	360 (100)

Table S3: Main results for ICU survivors and non-survivors versus controls.

	Controls mean±SD	**ICU survivor** mean±SD	**ICU non-survivor** mean±SD	**P-value Control vs ICU-survivor**	**P-value ICU-survivor vs non-survivor**
Plasma ACTH and cortisol time course					
Mean Plasma ACTH (pg/ml)	49.6±37.9	16.9±9.7	17.1±9.1	<0.001	0.83
Mean Plasma Total Cortisol (µg/dl)	11.9±2.3	16.8±7.8	17.3±8.7	0.01	0.73
Free Cortisol day 1 (µg/dl)	0.4±0.1	1.8±2.1	2.8±4.0	0.001	0.93
Free Cortisol day 7 (µg/dl)	0.4±0.1	1.3±1.3	1.4±0.8	<0.001	0.28
Cortisol kinetics during stable isotope tracer infusions					
Plasma Cortisol at steady state (µg/dl)	2.9±1.3	9.1±4.1	14.7±2.7	0.002	0.07
Rate of Appearance of Cortisol (mg/h)	1.5±1.5	2.6±1.4	3.7±0.4	0.05	0.28
Plasma Clearance of D4-cortisol (liter/min)	0.5±0.3	0.3±0.1	0.26±0.06	0.03	0.83
Plasma D4-cortisol (µg/dl)	1.7±1.1	2.7±1.0	2.3 0.6	0.03	0.83
Net rate of appearance Cortisone (mg/h per µg/dl)	0.14±0.07	0.07±0.02	0.07±0.0006	0.008	0.90
Rate of appearance of D3-cortisol (mg/h)	0.5±0.1	0.4±0.1	0.4±0.1	0.42	0.28
Plasma clearance of a therapeutic dose of cortisol					
Plasma Clearance of Cortisol (liter/min)	0.10±0.02	0.05±0.03	0.02±0.01	0.001	0.03
Cortisol Half-life (h)	1.8±0.3	9.4±10.0	17.9±13.0	<0.001	0.03
Activity of cortisol metabolizing enzymes estimated by ratios of urinary cortisol metabolites					
11β-HSD2 (E/F)	0.7±0.2	0.5±0.3	0.3±0.3	0.01	0.13
overall-11β-HSD (THF+allo/THE)	1.3±0.4	6.9±6.3	4.1±2.6	<0.001	0.35
5α-reductase (alloTHF/F)	4.9±2.6	2.3±1.8	0.5±0.3	0.002	0.01
5β-reductase (THF/F)	4.6±1.3	3.0±2.9	1.3±1.4	0.001	0.14
5β-reductase (THE/E)	11.4±3.7	2.6±2.2	2.9±2.5	<0.001	1.00
Tissue expression of cortisol metabolizing enzymes					
		NA			NA

Table S4: Main results for duration of stay in ICU at sampling

Plasma ACTH and cortisol time course		
NA		
Cortisol kinetics during stable isotope tracer infusions	**R^2 ***	**P-value**
Plasma Cortisol at steady state (µg/dl)	<0.001	0.96
Rate of Appearance of Cortisol (mg/h)	0.02	0.70
Plasma Clearance of D4-cortisol (liter/min)	0.10	0.34
Plasma D4-cortisol (µg/dl)	0.17	0.21
Net rate of appearance of Cortisone (mg/h per µg/dl)	0.15	0.24
Rate of appearance of D3-cortisol (mg/h)	0.15	0.24
Plasma clearance of a therapeutic dose of cortisol		
Plasma Clearance of Cortisol (liter/min)	0.003	0.81
Cortisol Half-life (h)	0.03	0.42
Activity of cortisol metabolizing enzymes estimated by ratios of urinary cortisol metabolites		
11β-HSD2 (E/F)	0.11	0.05
overall-11β-HSD (THF+alloTHF/THE)	0.04	0.23
5α-reductase (alloTHF/F)	0.007	0.63
5β-reductase (THF/F)	<0.001	0.94
5β-reductase (THE/E)	<0.001	0.94
Tissue expression of cortisol metabolizing enzymes		
5α-reductase mRNA levels liver	0.03	0.27
5α-reductase mRNA levels omentum	0.03	0.54
5α-reductase mRNA levels subcutaneous fat	0.008	0.75
5β-reductase mRNA levels liver	0.01	0.40
5β-reductase protein levels liver	0.01	0.39

** **R^2** for correlation between ICU day of sampling and the different variables listed*

Table S5: Main results for severity of illness (APACHE II)

Plasma ACTH and cortisol time course	**R^2 ***	**P-value**
Mean Plasma ACTH (pg/ml)	0.02	0.39
Mean Plasma Total Cortisol (µg/dl)	0.01	0.41
Free Cortisol day 1 (µg/dl)	0.009	0.53
Free Cortisol day 7 (µg/dl)	0.03	0.28
Cortisol kinetics during stable isotope tracer infusions		
Plasma Cortisol at steady state (µg/dl)	0.09	0.37
Rate of Appearance of Cortisol (mg/h)	<0.001	0.97
Plasma Clearance of D4-cortisol (liter/min)	0.06	0.45
Plasma D4-cortisol (µg/dl)	0.002	0.89
Net rate of appearance of Cortisone (mg/h per µg/dl)	0.11	0.30
Rate of appearance of D3-cortisol (mg/h)	0.07	0.41
Plasma clearance of a therapeutic dose of cortisol		
Plasma Clearance of Cortisol (liter/min)	0.11	0.15
Cortisol Half-life (h)	0.01	0.56
Activity of cortisol metabolizing enzymes estimated by ratios of urinary cortisol metabolites		
11β-HSD2 (E/F)	0.02	0.38
overall-11β-HSD (THF+allo/THE)	<0.001	0.97
5α-reductase (alloTHF/F)	0.07	0.13
5β-reductase (THF/F)	0.002	0.75
5β-reductase (THE/E)	0.08	0.10
Tissue expression of cortisol metabolizing enzymes		
	NA	NA

* ***R^2*** *for correlation between APACHE-II score and the different variables listed*

Table S6: Main results for unavoidable predisposing factors of adrenal dysfunction in critical illness

OPIOIDS	**Opioids (mean±SD)**	**No Opioids (mean±SD)**	**P-value**
Plasma ACTH and cortisol time course	N=28	N=19	
ACTH levels on day 7 (pg/ml)	21.7±21.2	26.4±18.5	0.06
Total Cortisol levels day 7 (µg/dl)	17.2±9.4	18.2±10.0	0.80
Free Cortisol levels day 7 (µg/dl)	1.3±1.3	1.3±1.1	1.00
Cortisol kinetics during stable isotope tracer infusions	N=10	N=1	
Plasma Cortisol at steady state (µg/dl)	10.0±4.6	11.3	1.00
Rate of Appearance of Cortisol (mg/h)	2.8±1.4	2.5	0.87
Plasma Clearance of D4-cortisol (liter/min)	0.3±0.1	0.2	0.42
Plasma levels D4-cortisol (µg/dl)	2.5±0.9	3.4	0.42
Net rate of appearance of Cortisone (mg/h per µg/dl)	0.08±0.02	0.05	0.15
Rate of appearance of D3-cortisol (mg/h)	0.4±0.1	0.3	0.26
Plasma clearance of a therapeutic dose of cortisol	N=15	N=5	
Plasma Clearance of Cortisol (liter/min)	0.03±0.02	0.05±0.04	0.33
Cortisol Half-life (h)	12.8±11.3	11.1±13.6	0.40
Activity of cortisol metabolizing enzymes by urinary metabolites	N=16	N20	
11β-HSD2 (E/F)	0.5±0.4	0.4±0.3	0.70
overall-11β-HSD (THF+allo/THE)	8.0±7.8	5.4±4.1	0.55
5α-reductase (alloTHF/F)	2.0±2.0	2.0±1.7	0.40
5β-reductase (THF/F)	2.6±3.3	2.8±2.3	0.62
5β-reductase (THE/E)	2.0±1.9	3.2±2.4	0.12
Tissue expression of cortisol metabolizing enzymes	N=34	N=10	
5α-reductase mRNA levels liver	0.7±1.9	0.3±0.2	0.87
5α-reductase mRNA levels omentum	0.9±0.8	1.5±1.4	0.43
5α-reductase mRNA levels subcutaneous fat	1.0±0.7	0.8±0.3	0.93
5β-reductase mRNA levels liver	0.3±0.8	0.2±0.3	0.61
5β-reductase protein levels liver	0.2±0.4	0.7±1.5	0.01
ANTI-COAGULANT THERAPY	**Therapeutic anticoagulants (mean±SD)**	**No therapeutic anticoagulants (mean±SD)**	**P-value**
Plasma ACTH and cortisol time course	N=5	N=42	
on ACTH levels day 7	22.8±10.1	23.7±21.1	0.58
on endogenous cortisol levels day 7 (yes vs. no)	17.1±8.8	17.6±9.8	0.95
Plasma CBG day 7 (mg/l)	45.7±5.7	47.6±12.1	0.78
Free Cortisol day 7 (µg/dl)	1.3±1.1	1.3±1.2	0.95
Cortisol kinetics during stable isotope tracer infusions			
	none on therapeutic anticoagulants		

Plasma clearance of a therapeutic dose of cortisol			
	none on therapeutic anticoagulants		
Activity of cortisol metabolizing enzymes estimated by ratios of urinary cortisol metabolites			
	none on therapeutic anticoagulants		
Tissue expression of cortisol metabolizing enzymes	**N=2**	**N=42**	
5α-reductase mRNA levels liver	0.3±0.2	0.6±1.7	0.87
5α-reductase mRNA levels omentum	3.0±0.0	0.9±0.8	0.13
5α-reductase mRNA levels subcutaneous fat	0.2±0.0	0.2±0.1	0.89
5β-reductase mRNA levels liver	0.1±0.2	0.3±0.7	0.80
5β-reductase protein levels liver	0.05±0.06	0.30±0.86	0.63

Most known predisposing factors for adrenal dysfunction were excluded. The only predisposing drugs that could not be excluded, as often required during critical illness, were anticoagulants and opioids. To check if they affect our conclusions, we looked for differences between treated and non-treated patients. We could confidently exclude potential confounding by such treatments.

Table S7: Results in relation to markers of organ hypoperfusion

D4-cortisol plasma clearance (liter/min)			
	R^2 *	**P-value**	
Cardiac Output (liter/min)	0.22	0.34	
Blood Lactate (mmol/l)	0.11	0.31	
24h Urine Output (liter)	0.02	0.66	
	Treatment	**No treatment**	**P-value**
	mean ±SD	**mean ±SD**	
Inotropes	0.27±0.19	0.25±0.07	0.88
Vasopressors	0.22±0.06	0.32±0.15	0.27
Opioids	0.26±0.11	0.17±0	0.42
Therapeutic anticoagulants	none on therapeutic anticoagulants		
Cortisol plasma clearance after a therapeutic hydrocortisone dose (liter/min)			
	R^2 *	**P-value**	
Cardiac Output (liter/min)	0.36	0.15	
Blood Lactate (mmol/l)	0.11	0.14	
24h Urine Output (liter)	0.10	0.15	
	Treatment	**No treatment**	**P-Value**
	mean ±SD	**mean ±SD**	
Inotropes	0.03±0.02	0.05±0.03	0.26
Vasopressors	All on vasopressors		
Opioids	0.03±0.02	0.05±0.04	0.33
Therapeutic anticoagulants	none on therapeutic anticoagulants		

**** R^2** for correlation between cardiac output, blood lactate or 24h urine output and cortisol plasma clearance in the tracer study and after a therapeutic hydrocortisone injection.*

Since cardiac output and organ perfusion may determine removal and metabolism of a substance, reduced cortisol clearance in critical illness could be influenced by a change in these parameters. Therefore, we looked to the association of indirect markers of organ perfusion with plasma cortisol clearance in the two studies. First, we analyzed whether cortisol clearance correlated with cardiac output. There was no correlation at all. Second, we looked whether cortisol clearance was associated with the need of treatment for low cardiac output (catecholamines such as adrenalin, dobutamine, isuprenaline or non-catecholamine inotropes such as phosphodiesterase inhibitors etc.) or with the need for vasopressors (cathecholamines such as noradrenalin or dopamine, or non-catecholamines such as vasopressin etc.). Again, there was no relation at all. Third, if the reduced cortisol clearance were the consequence of a general hypoperfusion state, one would expect also a correlation between cortisol clearance and plasma lactate (marker of global oxygen debt) or urine output (a marker of kidney perfusion). None of this was the case. Hence, reduced cortisol clearance in critical illness could not be explained by a general hypoperfusion state.

Table S8: Urinary metabolites by LC-MS/MS

Metabolite	**Patients (n=36)**	**Controls (n=15)**	**P-value**
	median (IQR)	median (IQR)	
F*	21.6 (12.9-30.8)	4.2 (2.5-6.1)	<0.001
E*	6.7 (4.1-10.1)	4.7 (3.1-6.7)	0.06
allo-THF*	1.5 (0.9-3.5)	1.5 (0.8-2.3)	0.48
THF*	3.8 (2.2-8.2)	4.2 (2.2-6.8)	0.99
THE*	6.7 (3.1-17.6)	15.2 (10.6-27.1)	0.004

**relative amounts (area/area internal standard)*

Table S9: Urinary metabolites by GC-MS

Metabolite	**Patients (n=36) median (IQR)**	**Controls (n=15) median (IQR)**	**P-value**
F (µg/24h)	886 (534-1620)	273 (188-333)	<0.001
E (µg/24h)	312 (252-424)	180 (145-211)	<0.001
allo-THF (µg/24h)	1155 (450-2842)	1198 (992-1422)	0.80
THF (µg/24h)	1779 (726-4629)	1362 (957-1493)	0.22
THE (µg/24h)	663 (328-1003)	2145 (1520-2426)	<0.001

CHAPTER 4

REDUCED NOCTURNAL ACTH-DRIVEN CORTISOL SECRETION DURING CRITICAL ILLNESS

Adapted from: *Boonen E, Meersseman Ph, Vervenne H, Meyfroidt G, Guïza F, Wouters PJ, Veldhuis JD, Van den Berghe G. Reduced nocturnal ACTH-driven cortisol secretion during critical illness. Am J Physiol Endocrinol Metab (in press)*

4.1 ABSTRACT

Background: During critical illness, cortisol metabolism was recently found to be reduced. We hypothesize that such reduced cortisol breakdown may suppress pulsatile ACTH and cortisol secretion via feed-back inhibition.

Methods: To test this hypothesis, nocturnal ACTH and cortisol secretory profiles were constructed by deconvolution analysis from plasma concentration time-series in 40 matched critically ill patients and 8 healthy controls, excluding diseases or drugs that affect the HPA-axis. Blood was sampled every 10 minutes between 21:00h and 06:00h to quantify plasma concentrations of ACTH and (free) cortisol. Approximate entropy, an estimation of process irregularity, cross-approximate entropy, a measure of ACTH-cortisol asynchrony, and ACTH-cortisol dose-response relationships were calculated.

Results: Total and free plasma cortisol concentrations were higher at all times in patients than controls (all $P<0.04$). Pulsatile cortisol secretion was 54% lower in patients than controls ($P=0.005$), explained by reduced cortisol burst mass ($P=0.03$) whereas cortisol pulse frequency ($P=0.35$) and non-pulsatile cortisol secretion ($P=0.80$) were unaltered. Pulsatile ACTH secretion was 31% lower in patients than controls ($P=0.03$), again explained by a lower ACTH burst mass ($P=0.02$), while ACTH pulse frequency ($P=0.50$) and non-pulsatile ACTH secretion ($P=0.80$) were unchanged. ACTH/cortisol dose-response estimates were similar in patients and controls. ACTH and cortisol approximate entropy was higher in patients ($P\leq0.03$), as were ACTH/cortisol cross-approximate entropy ($P\leq0.001$).

Conclusions: Hypercortisolism during critical illness coincided with suppressed pulsatile ACTH and cortisol secretion and a normal ACTH/cortisol dose-response. Increased irregularity and asynchrony of the ACTH and cortisol time-series supported non-ACTH-dependent mechanisms driving hypercortisolism during critical illness.

4.2 INTRODUCTION

The stress hormone cortisol is an essential component of the 'fight or flight' adaptation to the stress of illness and trauma. Whenever the human brain senses a stressful event, the HPA-axis is activated via the release of CRH from the hypothalamic paraventricular nucleus into the portal circulation, which stimulates the anterior pituitary corticotrophs to secrete ACTH in turn driving cortisol synthesis and secretion from the adrenal cortex. Feedback inhibition exerted by cortisol at the pituitary and the hypothalamic level controls its own release.

The release of ACTH and cortisol occurs predominantly in discrete pulses superimposed upon a non-pulsatile hormonal release with a slower diurnal pattern so that minimal levels are present during sleep and maximal levels around awaking.[1] Several groups have reported that in patients suffering from critical illness, an extreme example of physical stress, high circulating cortisol levels are present in the face of low rather than high ACTH plasma concentrations. This constellation is referred to as the 'ACTH-cortisol dissociation'[2-4] and has been interpreted as ACTH-independent factors driving cortisol release.[5]

We showed via a stable isotope tracer study that the elevated morning plasma cortisol concentrations during critical illness are only to a limited extent explained by increased cortisol production. In patients with excessive inflammation, this cortisol production was found to correlate with the plasma concentrations of pro-inflammatory cytokines. Critically ill patients not suffering from excessive inflammation did not show elevated cortisol production at all, although the plasma cortisol concentrations were as high as in patients with excessive inflammation. In contrast, cortisol metabolism was found to be reduced irrespective of the inflammatory status, via suppressed expression and/or activity of the A-ring reductases and of 11β-HSD2.[2]

In these previous studies, only single sample plasma ACTH concentrations were reported. This precludes analysis of ACTH secretory dynamics and of its relationship with cortisol secretion during critical illness. We hypothesized that reduced cortisol breakdown during critical illness can elevate circulating cortisol to levels that are able to suppress pulsatile ACTH secretion via feedback-inhibition. If the responsiveness of cortisol secretion to a given ACTH concentration is unaltered during critical illness, such suppressed pulsatile ACTH secretion could then proportionately reduce pulsatile cortisol release in the face of elevated plasma cortisol concentrations. Such an additional regulator of the HPA axis will expectedly increase the irregularity and asynchrony of the ACTH and cortisol time series.

To test this hypothesis, the dynamics of ACTH and cortisol secretion, analyzed via nocturnal time series of repeated (every 10 minutes) blood samples, were compared between critically ill patients and

demographically matched healthy control subjects. First, pulsatile and non-pulsatile ACTH and cortisol secretory profiles were constructed from the plasma concentration time series by deconvolution analysis.[6] Second, with a complementary analytical model, the cortisol secretory dose-response to ACTH was quantified by coupling effector concentrations to time-delayed glandular secretion rates.[7] Third, the presence of alternative regulators of ACTH and cortisol was appraised by the approximate entropy (ApEn) of the respective time series, an estimation of process irregularity, and by cross-ApEn, a quantitation of ACTH-cortisol asynchrony.[6] Finally, plasma concentrations of free cortisol and of the pro-inflammatory cytokines IL-6 and TNF-α were quantified and correlated with the deconvolved ACTH and cortisol secretion rates and with the (cross-)ApEn to assess their potential role as regulators of the HPA-axis dynamics during critical illness.

4.3 MATERIALS AND METHODS

Patient and study samples

Overnight serial blood samples were taken from critically ill patients and demographically matched healthy controls. Baseline characteristics are described in Table 1.[8,9] The study protocol and consent forms were approved by the Institutional Ethical Review Board of the KU Leuven (ML6625). The study was registered at International Standard Randomized Controlled Trial Number Register (Number ISRCTN49306926). Written informed consent was obtained from the patients' next of kin and from the healthy volunteers.

During the time frame of the study, consecutive adult (age ≥ 18y) patients were screened for eligibility on a daily basis, from the population present in 5 ICUs (total of 73 beds), both medical and surgical, in the University Hospitals of KU Leuven. Patients with diseases predisposing to HPA axis dysfunction or treated with drugs affecting the HPA-axis were excluded (glucocorticoids within the last 3 months, etomidate or azoles within the last 72 hours).[10]

Healthy men and women were selected based on BMI, age and gender, to match with the patient group. They were invited to participate after screening for exclusion criteria, as mentioned above. Before the start of the study each healthy volunteer was examined by a medical doctor to confirm a healthy physical and psychological condition. The volunteers were admitted to a room adjacent to the ICU, to control for environmental factors. Lights went out between 22:00h and 23:00h, ambulation was permitted to the lavatory only and eating or drinking was not allowed.

Undiluted 2 ml blood samples were withdrawn from an arterial line every 10 minutes during 9 hours (21:00h-06:00h), allowing detection of cortisol release episodes of which the timing is non-randomly coupled to that of prior and simultaneous ACTH release episodes.[11] For critically ill patients, blood was sampled via the arterial catheter already in place as part of standard intensive care management. In healthy control subjects an arterial catheter was inserted into the arteria radialis. A Venous Arterial blood Management Protection system (VAMP®, Edwards Lifesciences, Irvine, USA) was connected to the arterial catheter to allow sampling without unnecessary blood waste. Total blood loss per patient/control did not exceed 110 ml.

All samples were collected in pre-chilled EDTA-tubes and immediately placed on ice. After centrifugation (233g, 10 min) at 4°C, plasma samples were frozen at -20°C and stored at -80°C until further analysis.

Table 1: Characteristics of study patients and controls			
	Patients (N=40)	**Controls (N=8)**	**P-value**
Demography and anthropometry			
Gender (no. male (%))	30 (75)	6 (75)	1.00
Age (yr) (mean ± SD)	59.1 ± 16.9	55.6 ± 4.5	0.27
BMI (kg/m^2)[a] (mean ± SD)	25.9 ± 4.8	24.2 ± 2.3	0.14
Admission Characteristics			
Diagnostic group on admission (no. (%))			
Cardiovascular	9 (22.5)		
Respiratory/Esophageal-lung surgery	7 (17.5)		
Abdominal/Gastro-intestinal/Hepatic	19 (47.5)		
Other	5 (12.5)		
APACHE II [b] (mean±SD)	36 ± 5		
Patient characteristics at study time			
Day of Sampling (median (IQR))	3.5 (2.0-5.0)		
SIRS[c] (no. (%))	28 (70)		
Sepsis (no. (%))	18 (45)		
Treatment with catecholamines[d] (no. (%))	26 (65)		
Treatment with opioids[d] (no. (%))	30 (75)		
Treatment with anti-coagulants[d] (no. (%))	6 (15)		
Treatment with sedatives (no. (%))	10 (25)		
Treatment with hypnotics (no. (%))	13 (33)		
Plasma creatinine (mg/dl) (median (IQR))	1.1 (0.6-2.2)		
24h urinary volume (ml) (median (IQR))	1225 (750-1950)		
Creatinine clearance[e] (ml/min)	52.7 (22.5-98.3)		
Renal Replacement Therapy (no. (%))	4 (10)		
Clinical Outcomes			
Duration of stay in ICU (median (IQR))	14 (9.3-22.5)		
Hospital Mortality (no. (%))	7 (18)		

*[a]BMI: body-mass index. [b]Acute Physiology and Chronic Health Evaluation II (APACHE II) ranges from 0 to 71, with higher scores indicating greater severity of illness.[8] [c]SIRS: Systemic Inflammatory Response Syndrome, determined by the BONE criteria.[9] [d]Other drugs known to affect the HPA-axis were excluded:[10] glucocorticoids, other steroids, anti-steroid chemotherapy, etomidate, azoles, phenytoin, rifampicin, glitazones, imipramin, phenothiazine, phenobarbital. [e]creatinine clearance: (urinary creatinine level * 24h urinary volume)/(plasma creatinine level *1440) (missing values in 30% of patients).*

Plasma concentrations of ACTH and cortisol for deconvolution analysis

Total plasma cortisol concentrations were quantified with a high-specificity and high-sensitivity radioimmunoassay (Immunotech, Prague, Czech Republic). Plasma ACTH was quantified by a high-sensitivity and high-specificity double-monoclonal immunoradiometric assay (Brahms Diagnostics, Berlin, Germany).

Analyses were performed according to the manufacturer's instructions. For the determination of ACTH, 300 µl of plasma was used and one extra calibrator of a lower concentration (2.4 pg/ml) was prepared

by dilution of the lowest kit calibrator (4.8 pg/ml) with zero serum to increase sensitivity. All samples from one patient/control were processed in the same assay run.

Plasma free cortisol concentrations to estimate negative feedback inhibition on ACTH

Plasma CBG and albumin concentrations were quantified in samples taken on five time points spread over the time course of the study. Plasma CBG was quantified with a commercially available radioimmunoassay (Riazen, Louvain-la-Neuve, Belgium). Plasma albumin was measured by the bromocresol green method with the Quantitative Colorimetric Albumin Determination assay (BioAssay Systems, Hayward, CA).

Based on total plasma cortisol, CBG and albumin concentrations free cortisol levels were calculated with the Coolens method, previously validated in critical illness.[12,13] Since CBG and albumin have a long plasma half-life, repeated measurement of plasma concentrations is not meaningful. Therefore plasma concentrations of CBG and albumin were quantified at several time points spread over the study period (at 21:30h, 23:40h, 01:30h, 03:40h and 05:30h). With use of the Coolens method,[12] and using plasma CBG and albumin concentrations, free plasma cortisol concentrations were calculated.

<u>Coolens Formula:</u> *Free cortisol* = $\sqrt{Z^2 + \frac{T}{(1+N)K}} - Z$ *with* $Z = \frac{1}{2K} + \frac{G-T}{2\,(1+N)}$

With G = plasma CBG concentration (in µmol/l), T = plasma total cortisol concentration (in µmol/l), K = affinity of CBG for cortisol = $3.10^7 M^{-1}$ and N = ratio of albumin bound cortisol to unbound cortisol which is 1.74 for a normal albumin concentration of 43 g/l.

As albumin levels are affected by critical illness, we adapted the Coolens Formula for individual albumin concentrations based on the following rationale: N" = $\frac{1.74}{43}$ x individual albumin conc (g/l).

<u>Adapted Coolens Formula</u>

Free cortisol = $\sqrt{(0.0167 + (G\text{-}T)\frac{1}{2\,(1+N'')})^2 + T * \frac{1}{(1+N'')*K}}$ *-0.0167 + (G-T)* $\frac{1}{2\,(1+N'')}$

Plasma free cortisol concentration for the other time points of the study period was calculated with use of the average ratio of free over total plasma cortisol concentration in the 5 samples in which CBG and albumin was measured.

Plasma cytokine concentrations

Plasma TNF-alpha and IL-6 concentrations were quantified on a single morning sample with commercial ELISAs (Invitrogen, Camarillo, USA).

Data analysis of time series

In order to allow quantification of cortisol secretory profiles from plasma concentration time series, and to estimate the cortisol secretory response to any given ACTH concentration, total plasma cortisol concentrations were required.[6,7] Plasma free cortisol concentrations were determined as this is the active hormone fraction that mediates cortisol effects and thus also feedback inhibition.

ACTH and total cortisol plasma concentration time series were transformed into secretion profiles by using a multiple parameter deconvolution analysis implemented in Matlab (MathWorks Inc, Natick, USA).[14] This method is designed to extract the two overlapping signals; basal (non-pulsatile) secretion rate and intermittent secretory bursts (number and mass) from the raw time series, based on a fixed half-life. For ACTH a normal half-life was used for patients and controls.[15] For cortisol, a distinct half-life for patients and controls was used, as recently determined.[2] Mathematical coupling, or multi-parameter interdependence, is an important issue, and in older deconvolution methods limits parameter exactness. In the present model, as shown by comparison with earlier versions,[6] parameter estimation is good from known synthetic time series, due to the selection of a limited number of variables which are conceptually largely independent on a biological basis.

Dose-response estimates of ACTH-cortisol drive were calculated as recently described[7] and consist of a six-parameter logistic function, relating time-varying ACTH concentrations to delayed glandular cortisol secretion rates with allowable stochastic variability due to dynamical biological parameters. Specific dose-response outcome parameters are: (a) efficacy (asymptotic maximal ACTH-stimulated cortisol secretion rate); (b) potency (defined algebraically by a negative exponential term and functionally by the ACTH concentration driving half-maximal cortisol secretion (EC_{50})); (c) adrenal sensitivity (maximal positive slope of the ACTH-cortisol dose-response relationship). The six-parameter model contains two potencies (onset and offset) and a lag time (inflection point) to accommodate allowable down-regulation of ACTH drive represented by a right-shifted inhibitory adaptation of the dose-response process after the peak response to an ACTH concentration pulse.[16,17] As a consequence, in the two-potency model an onset EC_{50} and recovery or down-regulated EC_{50} can be calculated.

Approximate entropy (ApEn) and cross-ApEn were calculated as previously reported.[18,19] ApEn is the likelihood that runs of patterns that are close for a number of observations remains close on next incremental comparisons[20] and evaluates the consistency of subpatterns in sequential measurements and estimates the relative contribution of confounding factors, with a larger ApEn corresponding to greater irregularity and thus more confounders. Additionally, cross-ApEn appraises the asynchrony between the patterns of two time series, which is expected to increase when additional regulators are present. It can be calculated in two directions, the forward cross-ApEn appraising feedforward regulation

from ACTH to cortisol and the reverse cross-ApEn estimating feedback regulation from cortisol to ACTH.[18,21,22]

Statistical analyses

It was a priori decided to study 40 critically ill patients and 8 demographically matched healthy controls, in order to account for a larger variability in hormonal secretion rates among patients than among controls. With this sample size, a difference in pulsatile cortisol secretion of ±50% could be detected with a power of 80% and a two-tailed α-error level of 5%.

Statistical analyses were performed with JMP (version10.0) (SAS Institute Inc., Cary, USA). Data are presented as means±SD or medians (IQR), as appropriate. We used the nonparametric Wilcoxon rank-sum test for not-normally distributed data and unpaired Student's t-test for normally distributed data obtained after log-transformation where required. Comparison of proportions was done by chi-square-testing. Associations between parameters were analyzed with linear regression analysis, after transformation to obtain an approximate normal distribution when required. The Pearson determination (R^2) coefficient was calculated and its significance analyzed by Analysis of Variance. Two-sided P-values ≤0.05 were considered statistically significant. Data were presented as box-plots where boxes represent medians and IQR and whiskers are 10^{th} and 90^{th} percentile.

4.4 RESULTS

In healthy control subjects, diurnal rhythm was present for ACTH and cortisol, with higher plasma concentrations present in the early morning hours, while there was no diurnal rhythm detectable in patients (Figure 1). Plasma free and total cortisol concentrations were constantly higher in patients than in controls (all $P<0.04$) and ACTH concentrations were comparable in patients and controls until 04:50h after which they were lower in patients (all $P<0.04$) (Figure 1). In line with results from previous studies, morning (at 06:00h) ACTH levels were lower [12.4 pg/ml (6.6-19.2)] in patients than controls [27.5 pg/ml (17.0-30.6)] ($P=0.002$) and morning cortisol levels were higher in patients [12.9±7.7 µg/dl] than controls [9.4±2.1µg/dl] ($P=0.01$).

Plasma CBG concentrations were constantly lower in patients (29.5±9.7µg/ml) than in controls (41.8±5.4 µg/ml; $P<0.0001$). Moreover, plasma albumin concentrations were also lower in patients (2.8±1.2g/dl) than controls (4.7±0.4; $P<0.0001$) in all samples. Consequently, calculated free cortisol was 8-fold higher in patients [1.6 (0.7-2.2) µg/dl] than controls [0.2 (0.1-0.3) µg/dl; $P<0.0001$] (Figure 1).

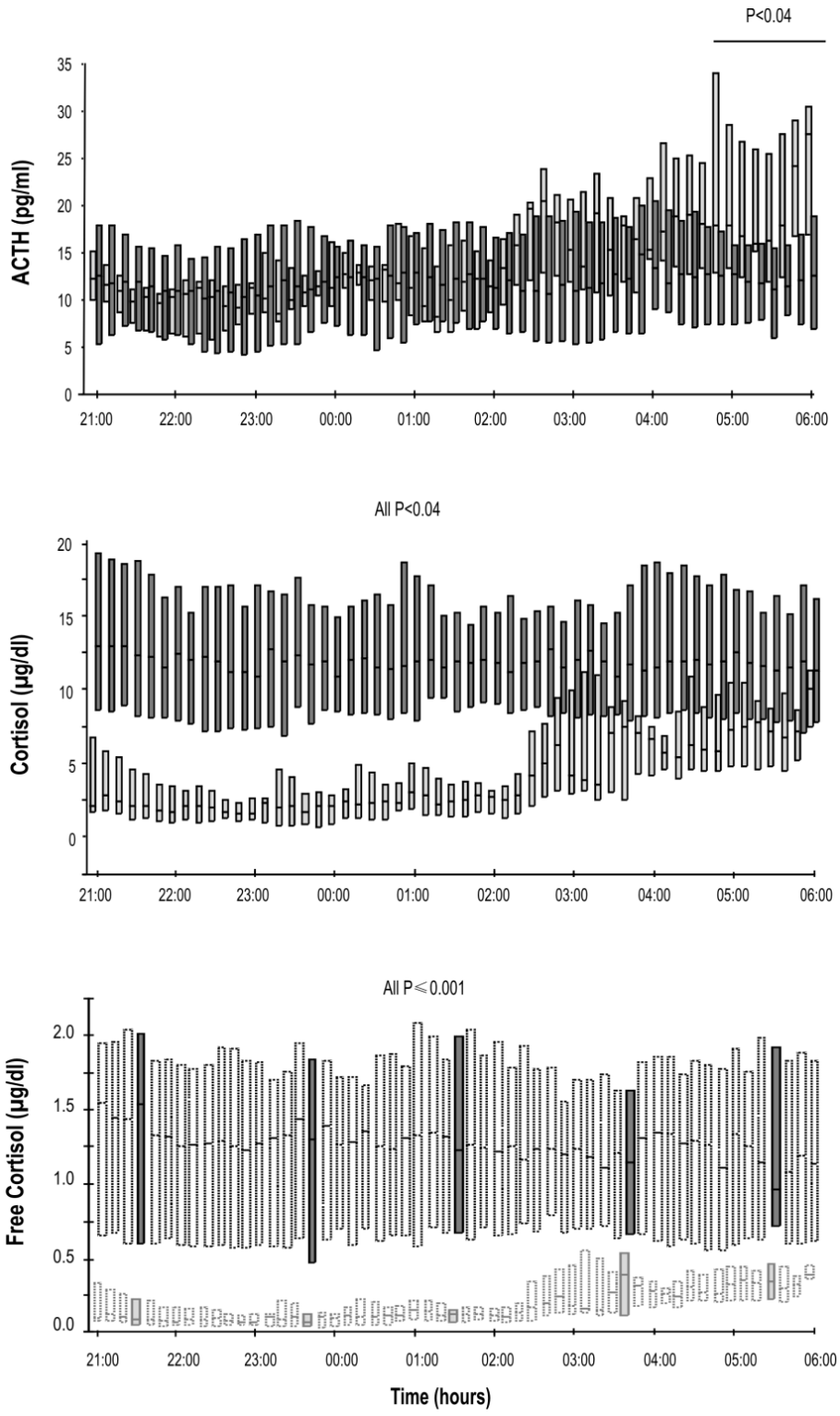


Figure 1 - ACTH and total and free cortisol plasma concentrations

Plasma ACTH and total cortisol concentrations in 40 patients (dark grey) and 8 controls (light grey) are depicted in the two upper panels, with boxplots representing medians and interquartile ranges. Plasma ACTH concentrations were lower in patients than controls from 04:50h onwards. Plasma total and free cortisol concentrations (lower panel) were always higher in patients than controls. Free cortisol was calculated with the Coolens method after determining plasma CBG and albumin concentrations at 21:30h, 23:40h, 01:30h, 03:40h, and 05:30h (filled bars in lower panel). From the average ratio free cortisol/total cortisol, free cortisol concentrations were estimated for the other time points (dashed bars in lower panel). P-values for group comparisons were determined by the Wilcoxon rank-sum test. For conversion of ACTH to SI units (pmol/l) multiply by 0.22. For conversion of cortisol to SI units (nmol/l) multiply by 27.6.

Deconvolution analysis of the ACTH time series revealed 31% lower pulsatile ACTH secretion in patients than controls (P=0.03), explained by a lower mass per ACTH burst (P=0.02) with unaltered ACTH pulse frequency (P=0.50), illustrated for one representative patient and one control in Figure 2.

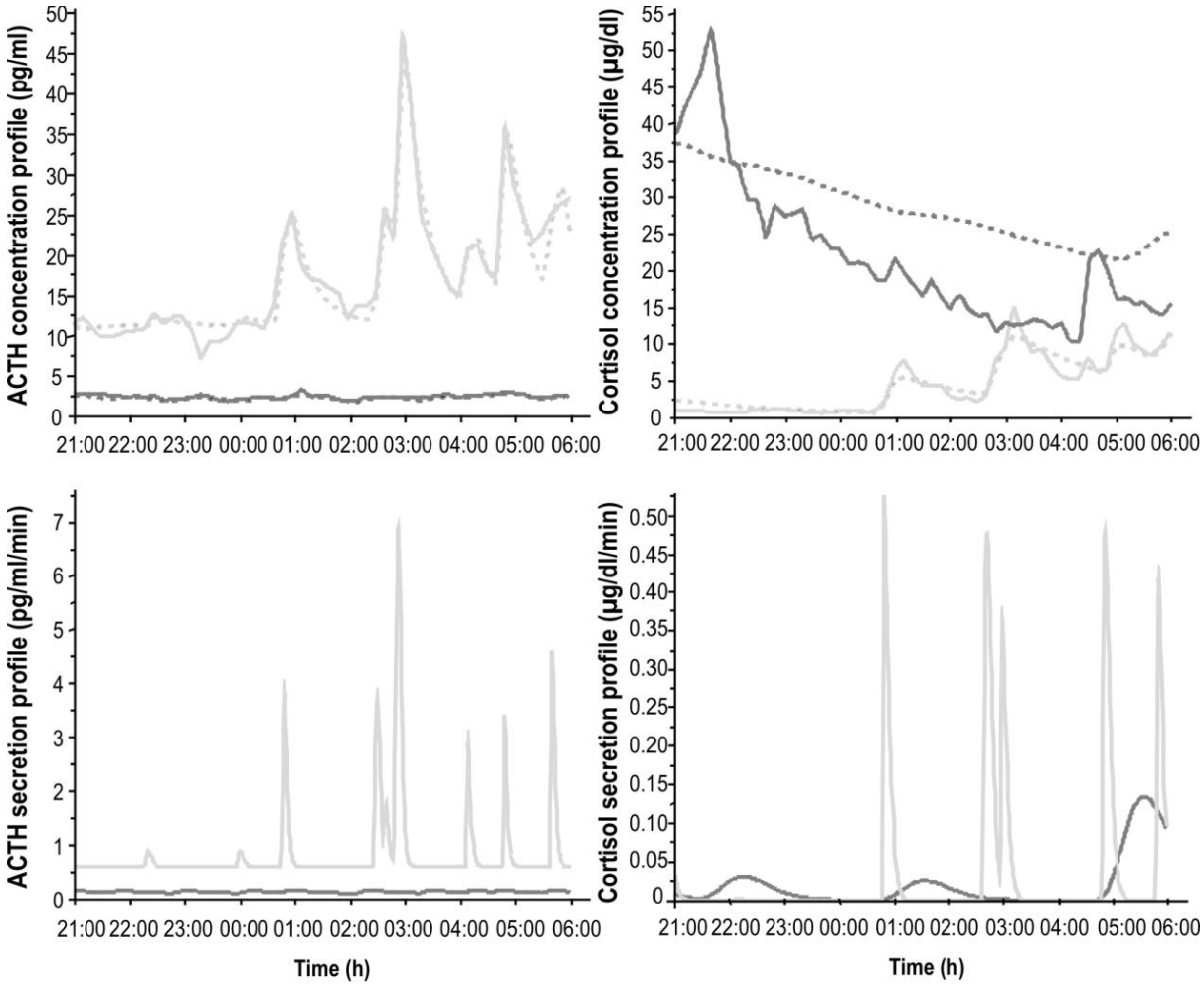


Figure 2 - Illustrative ACTH and cortisol profiles
Illustrative plasma ACTH and cortisol concentration time series obtained by blood sampling every 10 min from 21:00h to 06:00h in one patient (depicted in dark grey) and one control subject (depicted in light-grey). Top: Measured (continuous curves) and model-estimated (interrupted curves) plasma concentration profiles. Bottom: Estimated instantaneous secretion rates. For conversion of ACTH to SI units (pmol/l) multiply by 0.22. For conversion of cortisol to SI units (nmol/l) multiply by 27.6.

Non-pulsatile secretion was similar in patients and controls (P=0.80) (Figure 3 A-D). There was no correlation between plasma total or free cortisol concentrations and ACTH secretion.

Deconvolution analysis of the cortisol time series revealed 54% lower pulsatile cortisol secretion in critically ill patients than in controls (P=0.005), explained by a lower mass per cortisol burst (P=0.03) with a comparable cortisol pulse frequency (P=0.35), illustrated for one representative patient and one control in Figure 2. Non-pulsatile cortisol secretion was unaltered (P=0.80) (Figure 3 E-H). There was no correlation between cortisol secretion and plasma (total and free) cortisol concentrations.

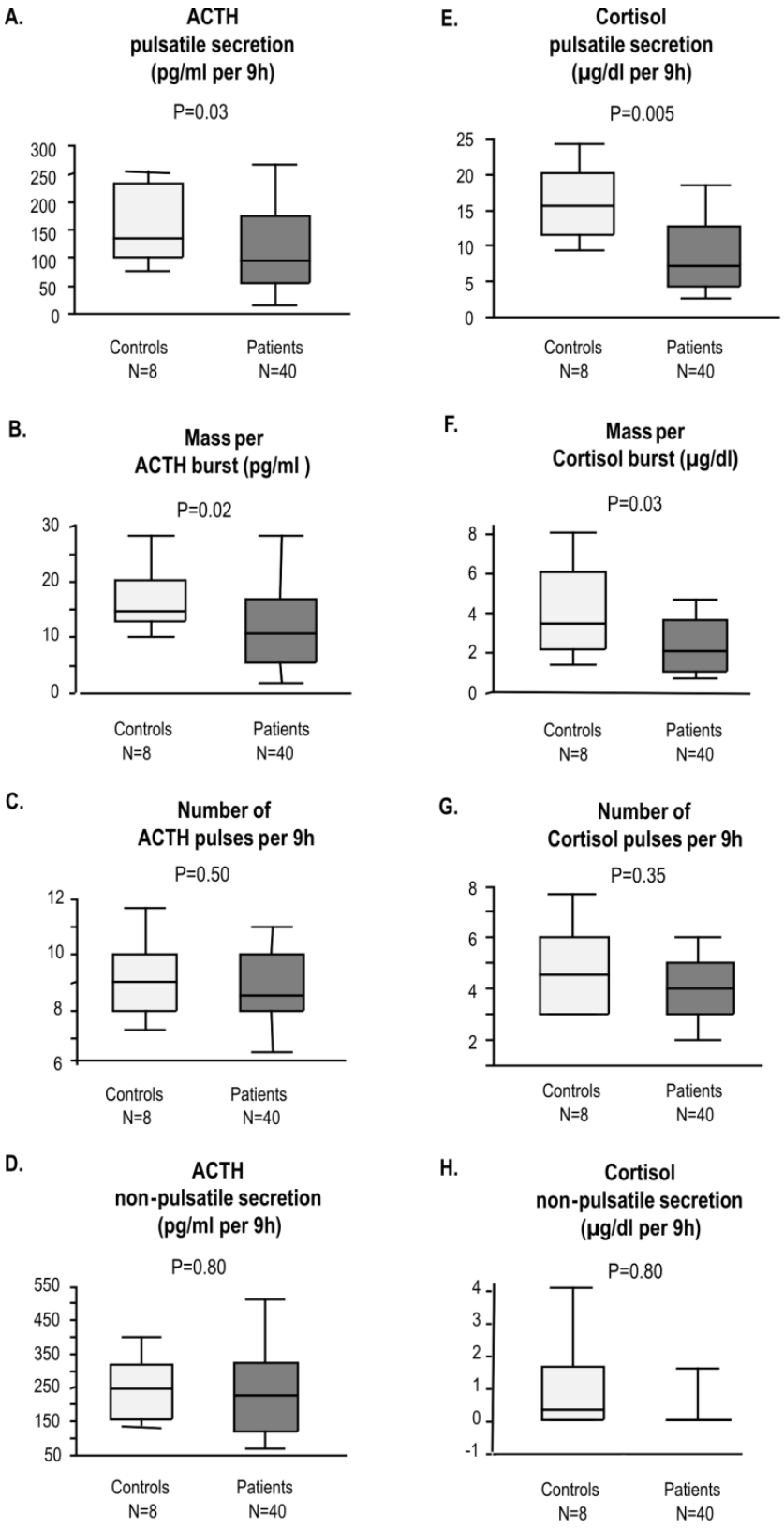


Figure 3 - ACTH and cortisol deconvolution results

Panels A and E represent pulsatile ACTH and cortisol secretion rates during the study period in patients (N=40) and in control subjects (N=8). Panels B and F depict the mass per ACTH and cortisol burst; panels C and G the number of ACTH and cortisol pulses and panels D and H the non-pulsatile ACTH and cortisol secretion rates during the study period. Boxes represent medians and interquartile ranges and whiskers are 10th and 90th percentile. P-values for group comparisons were determined by the Wilcoxon rank-sum test. For conversion of ACTH to SI units (pmol/l) multiply by 0.22. For conversion of cortisol to SI units (nmol/l) multiply by 27.6.

The plasma ACTH-concentration/cortisol-secretion dose response was similar in patients and controls (Table 2).

Table 2: ACTH and Cortisol Dose Response

	Patients (N= 40)	Controls (N=8)	P-value
Exponential Potency Onset	-7.7 (-12.6 - [-3.8])	-6.9 (-9.4 - [-3.9])	0.69
Exponential Potency Recovery	-19.4 (-39.3 - [-10.9])	-39.7 (-40.0 - [-18.8])	0.08
EC_{50} onset	15.3 (7.7 - 25.7)	9.0 (6.8 - 16.9)	0.15
EC_{50} recovery	34.1 (18.5-97.6)	44.2 (36.8-64.7)	0.40
Sensitivity	0.51 (0.19-1.25)	0.67 (0.39-0.91)	0.76
Efficacy (µg/dl per min)	0.75 (0.14-1.55)	0.24 (0.11-0.59)	0.11
Inflection point (10-min intervals*)	1.0 (1.0-3.0)	2.6 (1.0-2.9)	0.60

Data are expressed as median (IQR)
**number of 10-min samples between onset and offset of the cortisol response to an ACTH pulse*

The ApEn of the ACTH plasma concentration time series was 25% higher (P=0.03) and of cortisol 92% higher (P<0.0001) in patients than in controls. (Figure 4 A-B) The forward cross-ApEn was 37% higher (P=0.001) and the reverse cross-ApEn 68% higher in patients than in controls (P=0.0001) (Figure 4 C-D). Furthermore, the average plasma free cortisol concentration correlated positively with ACTH and cortisol ApEn (R^2=0.11; P=0.01 and R^2=0.32; P<0.0001 respectively) and with both cross-ApEns (Figure 4 E-F).

During critical illness, morning plasma concentrations of TNF-α and IL-6 were higher in patients [TNFα: 26.1 pg/ml (16.0-38.1) and IL6: 78.5 pg/ml (35.1-226.0)] than in healthy controls [TNFα: 8.8 pg/ml (6.2-11.9); P=0.0002 and IL6: 5.6 pg/ml (5.6-5.6); P<0.0001]. Both cytokine levels correlated inversely though weakly with pulsatile cortisol secretion (TNF-α: R^2=0.09; P=0.03 and IL-6: R^2=0.22; P=0.0009). Furthermore, plasma concentrations of both these cytokines correlated positively with cortisol ApEn (TNF-α: R^2=0.14; P=0.007 and IL-6: R^2=0.28; P<0.0001).

All the observed differences between patients and controls applied to survivors as well as non-survivors, and were not explained by variation in severity of illness, illness duration at sampling or by drugs such as opioids, catecholamines, insulin or anticoagulant treatment (data not shown).[10]

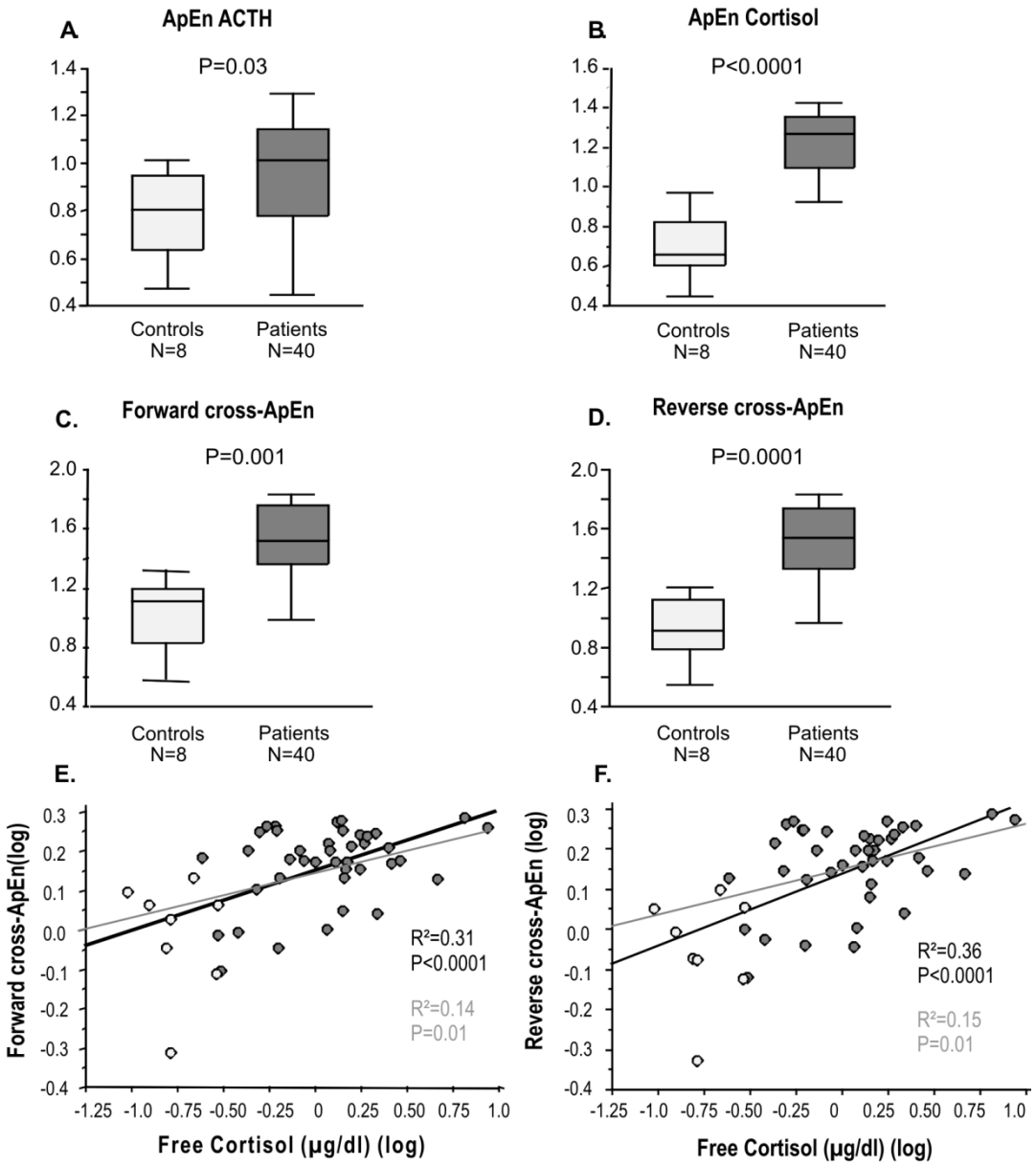


Figure 4 - ApEn and cross-ApEn for ACTH and cortisol system regularity

Panels A and B depict the ApEn of the ACTH and cortisol time series, with high levels indicating higher system irregularity, in patients (N=40) and in control subjects (N=8). Panel C depicts the cross-ApEn from ACTH to cortisol, a marker of feedforward regulation. Panel D depicts the cross-ApEn from cortisol to ACTH, a marker of feedback regulation. Boxes represent medians and interquartile ranges and whiskers are 10th and 90th percentile. P-values for group comparisons were determined by the Wilcoxon rank-sum test.Panel E and F depict the positive correlation between plasma free cortisol concentrations and the two cross-ApEn parameters in patients (dark grey) and control subjects (light grey) (black line). The positive correlation was maintained when analyzed among patients only (dark grey line). P-values for correlation were determined by Analysis of Variance.

4.5 DISCUSSION

We documented that hypercortisolemia during critical illness coincided with suppressed nocturnal pulsatile ACTH and cortisol secretion rates, whereas the cortisol secretory response to any given plasma ACTH concentration was unaltered. These findings speak against the classical dogma of an activated HPA axis in response to critical illness and instead suggest feedback-inhibition exerted by circulating cortisol. The increased irregularity and asynchrony of the ACTH and cortisol time series further support such non-ACTH-dependent mechanisms contributing to hypercortisolism during critical illness that is elevated through alternative pathways. These comprise reduced plasma clearance of cortisol and inferentially also altered splanchnic innervation and local factors within the adrenal glands driving cortisol release.[23]

Several studies have reported low plasma ACTH levels in the presence of elevated plasma cortisol in single samples of ICU patients, which has been referred to as the 'paradoxical ACTH-cortisol dissociation' of critical illness.[2-4] However, plasma hormone concentrations in single samples give little information about the dynamics of hormonal secretion. To document the dynamics of ACTH and cortisol secretion during critical illness, we transformed nocturnal plasma hormone profiles into deconvolution-derived hormonal secretion profiles, which demonstrated that pulsatile ACTH and cortisol secretion are both suppressed during critical illness. Also in septic rats, it was recently shown that after an initial activation suppression of ACTH occurs, possibly mediated by reduced orexin expression in the lateral hypothalamus.[24] In our study, suppressed pulsatile ACTH still appeared to drive the (smaller) cortisol pulses in critical illness. Indeed, the pulses of both ACTH and cortisol secretion were reduced in size but not in number, and the ACTH-cortisol dose-response relationships were maintained as during health.

Suppressed rather than increased nocturnal pulsatile ACTH and cortisol secretion during critical illness is surprising in relation to the classical understanding of the HPA axis stress response. It is generally assumed that critical illness, considered to be a condition of severe physical stress, results in several fold-increased cortisol production, an assumption that explains why doses of hydrocortisone given to substitute a failing adrenal gland during critical illness are more than 6-times higher (+/-200mg/day) than substitution doses for healthy subjects (+/-30mg/day).[25,26] We previously reported that the rate of appearance of cortisol, calculated with a stable isotope tracer infused during daytime, was only 1.8-fold higher in critically ill patients than in healthy matched controls, in the presence of low morning plasma ACTH values.[2] The current observation that overnight pulsatile cortisol secretion, calculated by deconvolution analysis, is not elevated at all during critical illness, and was actually lower than in healthy matched subjects, is likely explained by the loss of diurnal rhythm during critical illness. The nocturnal profiles indeed captured the early morning rise of ACTH and cortisol secretion in healthy subjects, which

was clearly absent in patients. Ideally, 24h profiles should be constructed in order to quantify the 24h differences between patients and controls. This, however, represents a major challenge in the ICU without disturbing the diagnostic and therapeutic activities that are required for the daytime care of these patients. However, the nocturnal cortisol secretion documented in this study was only half that of healthy controls. Furthermore, we previously showed that and as the cortisol production rate during the day, documented with isotopes, was not even a doubling of that of healthy subjects.[2] All together, it can be estimated that 24h cortisol production rates during critical illness may not be, or at best only moderately, increased.

The co-occurrence of elevated plasma total and free cortisol concentrations with suppressed ACTH and cortisol secretion rates implies that alternative regulators of cortisol dynamics must be involved during critical illness. This is corroborated by the lack of relation between plasma total and free cortisol levels and cortisol secretion, while (free) cortisol levels did correlate positively with the increased irregularity and asynchrony, as shown by increased ApEn and cross-ApEn values. Increasing evidence supports the existence of non-ACTH mediated regulation of cortisol production which involves cortico-medullary interactions, the neural input, the immune system, the vascular supply, growth factors and the intraglandular renin-angiotensin and CRH-ACTH systems.[27] This is further supported by the discovery of ectopic expression of membrane hormone receptors, which are functionally coupled to steroidogenesis by mimicking the cellular events triggered by ACTH receptor activation.[28]

Identifying the role of such non-ACTH dependent mechanisms in critical illness remains a challenge. Pro-inflammatory cytokines have been shown to drive cortisol secretion independently of ACTH and are known to be increased in critical illness.[29-31] In the current study the correlation between the elevated cytokine levels with the measures of irregularity and asynchrony might suggest such a role for cytokines, although the studied cytokines were found to correlate inversely, not positively, with cortisol secretion. A previous study also showed that injection of LPS reduces entropy measures, rendering a major role of circulating cytokines less likely.[32] Since cytokines are also produced locally within the adrenal gland by immune cells and adrenocortical cells, circulating levels alone may not account for the residual cortisol production. Next, the impact of sympatho-adrenomedullary system on the adrenal cortex was established in animal models showing that splanchnic innervation may directly drive cortisol secretion and stimulate the adrenal ACTH-sensitivity.[33,34] Critical illness is hallmarked by an increased sympathetic tone.[35] However, we observed that the adrenocortical sensitivity to ACTH was unaltered during critical illness. Also, exogenous catecholamines did not affect cortisol secretion rendering a major role of catecholamines less likely. However, adrenal nerves are catecholaminergic and peptidergic and these store, besides catecholamines, a wide variety of neuropeptides, including opioid peptides,

neuropeptide Y, vasoactive intestinal polypeptide, CRH and substance P.[27] The role of locally active neuropeptides during critical illness should be further investigated.[23] In addition, cortisol sensitive and insensitive neural pathways could play a role.[36-38] Finally, the adrenal medulla and intra-adrenal immune cells are a source of extrahypothalamic CRH and extrapituitary ACTH, which could also account for the residual cortisol production without an increase in circulating ACTH levels. However, a stimulatory effect on cortisol secretion of such local factors was not supported by the presented data as nocturnal cortisol secretion was lower, not higher in patients than in healthy controls.

The current observations of a reduced pulsatile ACTH secretion and a reduced pulsatile cortisol secretion, in the presence of elevated plasma levels of cortisol could be a consequence of the substantially suppressed metabolic clearance of cortisol during critical illness.[2] Elevating circulating cortisol by reduced breakdown may cause a tonic negative feedback inhibition on ACTH secretion. Such a mechanism would predominantly affect ACTH pulsatility via reducing the mass per ACTH pulse,[39-41] as observed here, in turn leading to suppressed pulsatile cortisol secretion.

The loss of pulsatility in the ACTH and cortisol secretory profiles observed during critical illness may have important implications. Indeed, the pulsatile nature of cortisol secretion prevents desensitization of transcriptional responses in target tissues and the pulsatile nature of ACTH release provides more effective signaling for the activation of the adrenal cortex than a constant ACTH stimulus.[42-44] However, it is unknown whether it is the ACTH pulse amplitude or the pulse frequency that determines adrenocortical responsiveness. As ACTH pulse frequency during critical illness was unaltered, and the amplitude of the ACTH pulses was not completely lost, it is unclear whether the latter suffices to maintain adrenal cortex integrity/functionality or could contribute to adrenal atrophy in the prolonged phase of critical illness. Likewise, one could speculate that the loss of pulsatility in the cortisol secretory profiles may play a role in the tissue-specific cortisol resistance observed during critical illness.[45,46] This requires further investigation.

The previous studies on the relationship between ACTH and cortisol during critical illness have reported only single sample measurements, which precluded conclusions on the dynamics of the secretory profiles and the coupling between the two hormones. A strength of our study is that it used time series of sampling every 10 minutes for 9 hours, previously found to allow accurate analysis of such dynamics.[11] Interestingly, we found that the low values for ACTH in the morning blood samples were quite representative for the suppressed pulsatile ACTH secretion. The presented study also has some limitations. First, the study was performed overnight only, which precludes robust conclusions about the 24h secretory profiles, as elaborated on above. Second, although a normal night atmosphere was

pursued, healthy controls may have experienced a certain stress level because of the arterial line, the ICU context, and a disrupted sleep pattern evoked by frequent blood sampling.

In conclusion, nocturnal pulsatile secretion of ACTH and cortisol was found to be jointly suppressed during critical illness with a normal ACTH-cortisol dose response. The increased irregularity and asynchrony of the ACTH and cortisol time series further support non ACTH-dependent mechanisms driving hypercortisolism during critical illness.

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CHAPTER 5

IMPACT OF DURATION OF CRITICAL ILLNESS ON THE ADRENAL GLANDS OF HUMAN INTENSIVE CARE PATIENTS

<u>Adapted from:</u> Boonen E, Langouche L, Janssens J, Meersseman Ph, Vervenne H, De Samblanx E, Pironet Z; Van Dijck L, Vander Perre S, Derese I, Van den Berghe G. Impact of duration of critical illness on the adrenal glands of human intensive care patients (Manuscript submitted for publication)

5.1 ABSTRACT

Background: Adrenal insufficiency is considered to be prevalent among critically ill patients, although the pathophysiology, diagnostic criteria and optimal therapeutic strategy remain controversial. During critical illness, reduced cortisol breakdown contributes substantially to elevated plasma cortisol and low plasma ACTH concentrations. Considering the trophic impact of ACTH on the adrenal cortex, we hypothesized that with longer duration of critical illness, subnormal ACTH adrenocortical stimulation may occur, which may predispose to adrenal insufficiency.

Methods: Adrenal glands were harvested ≤24h of death from 13 patients who died after a long ICU-stay [median 16 IQR (13-21) days], from 27 patients who died after a short ICU-stay [2 (1-5) days] and from 13 control subjects who died suddenly out-of-hospital. Prior glucocorticoid treatment was excluded. Microscopic adrenocortical zonational structure was evaluated by H&E staining. The amount of adrenal cholesterol-esters was determined by Oil-Red-O staining and mRNA expression of ACTH-regulated steroidogenic enzymes was quantified after mRNA quality control.

Results: The adrenocortical zonational structure assessed by H&E staining was disturbed in patients as compared with controls ($P<0.0001$), with indistinguishable adrenocortical zones only present in long ICU-stay patients ($P=0.003$ vs. controls). Adrenal glands from long ICU-stay patients, but not those of short ICU-stay patients, contained 21% less protein ($P=0.03$) and 9% more fluid ($P=0.01$) than those from controls, while they tended to weigh less for a comparable adrenal surface area. There was 78% less Oil-Red-O staining in long ICU-stay patients than in controls ($P=0.03$) and also 78% less than in short-stay patients ($P=0.03$), the latter similar to controls ($P=0.31$). The mRNA expression of MC2R, SCARB1, HMGCR, STAR and CYP11A1 was at least 58% lower in long ICU-stay patients than in controls (all $P\leq0.03$), and of MC2R, SCARB1, STAR and CYP11A1 at least 53% lower than in short ICU-stay patients (all $P\leq0.04$), whereas gene expression in short-stay patients was similar to controls.

Conclusions: Depletion of adrenocortical cholesterol esters and reduced expression of ACTH-regulated steroidogenic genes in prolonged, but not acute, critical illness suggest that sustained lack of ACTH-effect may contribute to risk of adrenal insufficiency in long-stay ICU patients.

5.2 INTRODUCTION

Critical illness, an example of severe physical stress, is hallmarked by increased circulating levels of the stress hormone cortisol. We have shown that, in contrast to previous understanding, these high levels are to a large extent explained by reduced cortisol breakdown, while cortisol production is only moderately increased, if at all.[1] Furthermore, plasma ACTH was shown to be low throughout at least the first week of critical illness,[1] possibly explained by negative feedback inhibition. Whereas reduced cortisol breakdown could be interpreted as a beneficial adaptation of the body to maintain hypercortisolemia in an "economic" way -- limiting the need for energy-consuming cortisol production in times of low energy availability -- sustained low ACTH levels could negatively affect structure and function of the adrenal cortex. Indeed, ACTH exerts important trophic and structural effects on the adrenal cortex[2] and depletion of ACTH in experimental models causes adrenal atrophy.[3] Furthermore, ACTH is responsible for both short- and long-term regulation of steroidogenesis.[4]

Cortisol cannot be stored in the adrenal gland, so its availability depends on rapid synthesis within the adrenal gland in case of stress. Cortisol is synthesized from cholesterol in the zona fasciculata of the adrenal cortex. The principal source of cholesterol for the adrenal glands is circulating low-density lipoprotein (LDL) cholesterol and to a lesser extent high-density lipoprotein (HDL) cholesterol, which is taken up via the LDL-receptor (LDLR) and the HDL-receptor [scavenger-receptor class B, member 1 (SCARB1)] respectively. Twenty percent of the cholesterol required for adrenal cortisol synthesis is newly produced within the adrenal cortex via 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR).[5] The cholesterol molecules are stored as cholesterol esters in intracellular vesicles, where they reside until steroidogenesis starts. Whenever more cortisol is needed, a cholesterol esterase rapidly mobilizes cholesterol out of the lipid droplets to the cytoplasm, from where it is transported to the inner membrane of the mitochondria (IMM).[5,6] This transport is mediated by the steroidogenic acute regulatory protein (STAR).[7,8] Within the IMM, cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone, which is the rate-limiting step in steroidogenesis. Afterwards, multiple enzymes regulate the further conversion of pregnenolone to cortisol.[7]

Within minutes after onset of stress, ACTH acutely activates its receptor, the melanocortin 2 receptor (MC2R), on the adrenal cortex which causes the release of cholesterol from the lipid droplets and increases STAR expression.[4] The more long-term impact of ACTH on the adrenal cortex involves increased transcription and translation of genes encoding proteins for cholesterol uptake (SCARB1, LDLR), for cholesterol synthesis (HMGCR) and for steroidogenesis (STAR and CYP11A1)] as such enhancing the synthetic capacity of the cells by providing a reserve pool for acute steroid

demand.[4,5,7,9,10] In addition, ACTH has a direct stimulatory effect on the expression of its own receptor (MC2R) which amplifies the adrenal responsiveness to ACTH.[11] Considering the extensive acute and chronic impact of ACTH on the adrenal cortex, persistently low ACTH concentrations profoundly affect the structure and function of the adrenal cortex. This is illustrated by the phenotype of pro-opiomelanocortin (POMC)-deficient mice, characterized by loss of adrenocortical zonational structure, adrenocortical lipid depletion, reduced ACTH signaling and adrenal atrophy.[3,12]

We hypothesized that low plasma ACTH concentrations during prolonged critical illness are associated with reduced trophic ACTH effects on the adrenal cortex, reflected by less lipid droplets and reduced expression of ACTH-regulated enzymes. Such effects could predispose to symptomatic adrenal failure in the prolonged phase of critical illness.[13] To test this hypothesis, we studied adrenal glands, harvested postmortem, from patients who died in the ICU after a short or a prolonged critical illness and compared these with adrenal glands from individuals who died acutely out-of hospital. We quantified alterations in size, weight and microscopic structure of the adrenal glands, the adrenocortical storage of cholesterol-esters and mRNA expression of different key ACTH-regulated proteins of the steroidogenic pathway.

5.3 MATERIALS & METHODS

Patients and study samples

From patients who died in the ICU and for whom an autopsy was requested by the attending physician, adrenal glands were harvested for this study. Patients who were on chronic glucocorticoid treatment during the preceding 3 months or who had received acute glucocorticoid treatment within 7 days prior to the day of death were excluded. For comparison, adrenal glands were harvested at the occasion of a planned autopsy from individuals who suddenly died out-of-hospital, further referred to as "control subjects", in collaboration with the emergency and forensic departments of the Leuven University Hospital. Control subjects who were known to suffer from chronic illnesses or were treated with glucocorticoids were excluded. All adrenal glands were harvested within 24h of death.

The Leuven University Hospital permits postmortem tissue sampling for academic purposes whenever a patient or his/her legal representative consented upon hospital admission. This is performed via a hospital-wide information and consent procedure, requiring active opting-out when not consenting, with opting-out remaining possible until time of death. The study protocol was approved by the Institutional Ethical Review Board of the KU Leuven (ML6625). The study was registered at International Standard Randomized Controlled Trial Number Register (Number ISRCTN49306926).

We wanted to investigate the impact of duration of critical illness. To this end, control subjects who died suddenly out-of-hospital were compared with patients who died after a long ICU-stay (>7days) and with patients who died after a short ICU-stay (≤7 days). The sample size was determined by the *a priori* calculated need of at least 13 patients in each group, in order to detect a difference in lipid staining of about 50% in long ICU-stay patients, with a power of 80% and certainty of 95%. Harvesting continued until this minimal number was reached and all patients who did not have any exclusion criteria and from whom adrenal glands were harvested within 24 hours after death were kept in the study.

Processing of adrenal glands

Immediately after harvesting, adrenal glands were processed for the different analyses. After cleaning from adherent fat, adrenal glands were weighed and photographed to estimate surface area with Image J 1.44p software (Wayne Rasband). Adrenal tissue intended for paraffin embedding was immediately placed in 6% paraformaldehyde. Adrenal tissue intended for cryostat sectioning were snap-frozen in liquid nitrogen cooled isopentane, while tissue intended for gene expression was immediately snap-frozen in liquid nitrogen and stored at -80°C until the time of analysis. All the analyses were performed on the left adrenal gland of each patient.

To quantify water content, approximately 50mg of adrenal gland tissue was weighed before and after 20h of lyophilisation (SpeedVac Concentrator, Thermo scientific). The difference in weight divided by the start weight represented the water fraction of the tissue. To determine the total protein content, adrenal samples (approximately 100mg) were weighed and homogenized in 4x NP40-glycerol lysis buffer with phosphatase and protease inhibitors, using ceramic beads and a Precellys 24 homogenizer (6000 rpm, 45 sec, Bertin Technologies, Villeurbanne, France). Protein content was quantified using Coomassie Protein Assay Reagent (Pierce Biotechnology Inc., Rockford, USA).

Microscopic analysis of the adrenocortical zonational structure

After embedding in paraffin, 5 µm-thick sections were stained with hematoxylin and eosin (HE). To evaluate adrenocortical zonational structure, HE-sections were scored semi-quantitatively based on the identification of the three zones in the adrenal cortex at a 5 x magnification using a Leica DM3000 microscope. Two investigators, blinded for group allocation, evaluated all sections separately and any discrepancy between them was resolved by consensus. Adrenocortical zonational structure was graded as follows: '0' when the three adrenocortical zones were clearly distinguishable, '1' for those with a moderately distorted zonational structure and '2' when the different zones were no longer distinguishable.

Quantification of cholesterol-ester storage in the adrenal glands

To quantify cholesterol-esters stored in the adrenal gland, Oil red O (ORO) staining was performed on 10µm-thick frozen tissue sections. ORO is a red dye used for staining neutral lipids and therefore stains the cholesterol esters in the lipid droplets of the adrenal cortex. Sections were dried at room temperature, fixed in propylene glycol and stained overnight in 0.5% ORO (Sigma Aldrich, St.Louis, USA) in propylene glycol and counterstained with hematoxylin.

Digital microscopic images of the ORO stainings were analyzed for the amount of redness per surface unit and the intensity of this redness with an in house developed computer algorithm from a plug-in for ImageJ 1.44p (Wayne Rasband). In short, the stained areas were quantified by training a classification algorithm to detect staining in the brightness and color saturation channels of the digitized images. This allows the differently stained areas to be extracted from the overall image, after which their respective surface areas can be calculated by the number of image pixels present in them. As a pre-processing step, image artifacts such as dust spots or staining drops were removed manually. For the intensity of the red-staining, the maximal and minimal redness (interpreted as pixel saturation) was determined for each individual image and labeled respectively 1 and 0. Every pixel was evaluated against this scale.

The mean of all values for all pixels represented the intensity of the staining. The product of the intensity and amount of redness per tissue surface was considered to represent the total amount of available cholesterol esters.

mRNA expression of ACTH-regulated key proteins of the steroidogenic pathway

Total mRNA was extracted from adrenal gland tissue (approximately. 30mg) using Qiazol lysis reagent (Qiagen, Hilden, Germany) and subsequently purified with RNAeasy Mini columns (Qiagen). Samples were treated with DNase to remove all genomic DNA, and 1µg total mRNA was reverse-transcribed using random hexamers. cDNA levels of genes regulated by ACTH signaling [for cholesterol uptake and synthesis (SCARB1, LDLR and HMGCR) and crucial genes for steroidogenesis (MC2R, STAR and CYP11A1)] were quantified in real time with the TaqMan® gene expression assays using the StepOnePlus™ System (Applied Biosystems, Carlsbad, USA) (Hs00196245_m1, 4310884E, Hs02758991_g1, Hs03928985_g1, Hs00167984_m1, Hs00986559_g1, Hs00300820_s1, Hs00969821_m1, Hs00181192_m1, Hs00168352_m1). Individual samples with a copy number coefficient of variation greater than 20% were reanalyzed. Data were expressed relative to expression 18S ribosomal 5 RNA (RNA18S5), a housekeeping gene of which the expression was not altered neither by critical illness nor by the duration of illness, and as a fold difference from the mean of the control subjects.

To exclude confounding effects of RNA degradation that may have occurred during the postmortem period, mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was evaluated with two different primer sets designed to amplify fragments at two distinct exons, (exon 6-7 and exon 3). To evaluate the relative proportion of the adrenal gland medulla in the tissue samples, cDNA levels for neurofilament light-polypeptide (NEFL), a marker for neuronal elements, were also quantified.

Statistical analyses

Statistical analyses were performed with JMP (version10.0) (SAS Institute Inc., Cary, USA). Data are presented as means±SD or medians with IQR, as appropriate. All experimental results were analyzed with a nonparametric Wilcoxon singed rank test. Comparison of proportions was done by chi-square-testing. Associations between parameters were analyzed with linear regression. The Pearson determination (R^2) coefficient was calculated and its significance analyzed by Analysis of Variance. No corrections were made for multiple comparisons. The *a priori* defined primary comparison was that between long ICU-stay patients and control subjects, as this is where the hypothesized differences were expected. Two-sided P-values <0.05 were considered statistically significant.

5.4 RESULTS

The study comprised adrenal glands harvested from 13 long-stay ICU patients, 27 short ICU-stay patients and 13 control subjects. The characteristics of patients and control subjects are described in Table 1.[14,15]

Weight, size and microscopic zonational structure of the adrenal glands

The weight of the adrenal glands, corrected for body weight, was not different among the groups [median 0.10 IQR (0.07-0.12) g/kg in the control subjects, 0.10 (0.08-0.12) g/kg in the short ICU-stay patients and 0.08 (0.06-0.10) g/kg in the long ICU-stay patients (all Ps>0.08)]. Also the adrenal surface area was similar for all groups [10.2 (8.8-12.2) cm² in control subjects, 11.2 (9.1-13.7) cm² in the short ICU-stay patients and 11.1 (9.3-16.5) cm² in the long ICU-stay patients (all Ps>0.25)].

The fraction water in adrenal glands from long ICU-stay patients [76% (IQR 72-78)] was larger than in those from control subjects [69% (62-74); P=0.01], while no difference was observed between short ICU-stay patients [72% (IQR 67-76)] and controls (P=0.22) or between the 2 ICU patient groups (P=0.08). There was 21% less protein per milligram tissue in adrenal glands from long ICU-stay patients [74µg/mg (IQR 67-78)] than in those from control subjects [94µg/mg (IQR 71-101), P=0.03]; and also 23% less protein in short ICU-stay patients [72µg/mg (IQR 66-83)] than in control subjects (P=0.02). Adrenal protein content in both ICU patient groups was similar (P=0.89).

Microscopic semi-quantitative scoring revealed disturbed adrenocortical zonational structure in both ICU patient groups as compared with control subjects (P<0.0001) (Figure 1). As compared with control subjects, indistinguishable zonational structure was significantly more often present only in the long ICU-stay patients (P=0.003).

Quantification of adrenal cholesterol-ester storage

Computerized quantification of the ORO staining revealed that in adrenal glands from long ICU-stay patients, there were 78% less cholesterol esters stored than in those from controls (P=0.03) and also 78% less than in those from short ICU-stay patients (P=0.03), while short ICU-stay patients were not different from controls. (Figure 2)

Table 1: Characteristics of ICU patients and control subjects

	Controls (N=13)	Short ICU-stay patients (N=27)	Long ICU-stay patients (N=13)	P-value Controls versus short ICU-stay patients	P-value Controls versus long ICU-stay patients	P-value short versus long ICU-stay patients
Demography and anthropometry						
Gender (no. male (%))	9 (69)	14 (52)	9 (69)	0.29	1.0	0.29
Age (yr) (mean ± SD)	40.8 ± 17.5	69.7±12.6	67.6±14.1	<0.0001	0.0017	0.62
BMI (kg/m^2) (mean ± SD)	24.9 ± 4.8	26.0 ± 5.5	25.8 ± 3.9	0.34	0.40	0.86
Admission Characteristics						
Diagnostic group on admission (no. (%))	N.A.					0.92
Cardiovascular		7 (26)	3 (23)			
Respiratory/Esophageal-lung surgery		10 (37)	4 (31)			
Abdominal/Gastro-intestinal/Hepatic		6 (22)	3 (23)			
Other		4 (15)	3 (23)			
APACHE II (mean±SD)	N.A.	34.6± 9.9	34.8± 6.0			0.70
SIRS (no. (%))	N.A.	27 (100)	12 (92)			0.14
Sepsis (no. (%))	N.A.	18 (67)	11 (85)			0.23
Patient characteristics at study time						
Cause of death						
Refractory Shock	N.A.	12 (44)	0 (0)			0.004
Therapy withdrawal for futility	N.A.	15 (56)	13 (100)			
Sudden out of hospital death*	13 (100)	N.A.	N.A.			
SIRS (no. (%))	N.A.	26 (96)	12 (92)			0.58
Sepsis (no. (%))	N.A.	16 (59)	11 (85)			0.10
Duration of stay in ICU prior to death (days) (median (IQR))	N.A.	2 (1-5)	16 (13-21)			<0.0001
Time interval between death and autopsy (h) (mean ± SD)	17.5 ± 4.5	15.0±6.8	15.4±4.8	0.28	0.31	0.79

*The body-mass index (BMI) is the weight in kilograms divided by the square of the height in meters. Acute Physiology and Chronic Health Evaluation II (APACHE II) ranges from 0 to 71, with higher scores indicating a greater severity of illness.[14] SIRS stands for Systemic Inflammatory Response Syndrome and is determined by the BONE criteria.[15] *Sudden out-of-hospital deaths comprised: Trauma (N=2), Cardiac Arrest (N=4), Drowning (N=2), Electrocution (N=1), Gun Shot (N=1), Acute Hemorrhage (N=1), Pulmonary Embolism (N=1), Sudden unexpected death of epilepsy (N=1).*

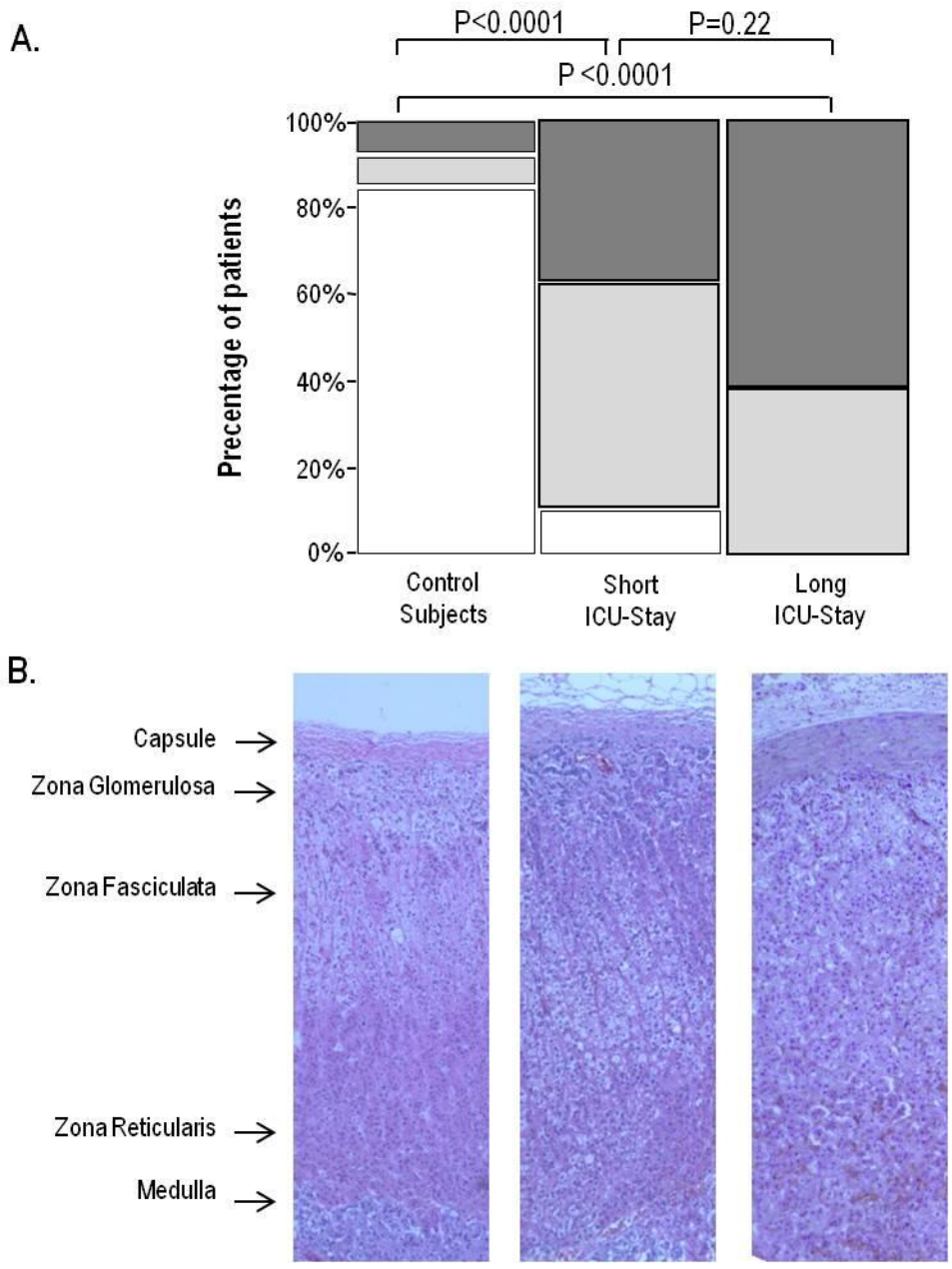

Figure 1: Semi-quantitative scoring of the loss of adrenocortical zonational structure

Panel A: The fraction of patients with score 0, indicating a normal zonational structure, is depicted in white. The fraction of patients with score 1, indicating a moderately distorted zonational structure, is depicted in light grey. The fraction of patients with score 2, indicating that the different zones were no longer distinguishable, is depicted in dark grey.

Panel B: The illustrations are representative examples of HE staining images for each group. Photographs were taken at a 5X magnification.

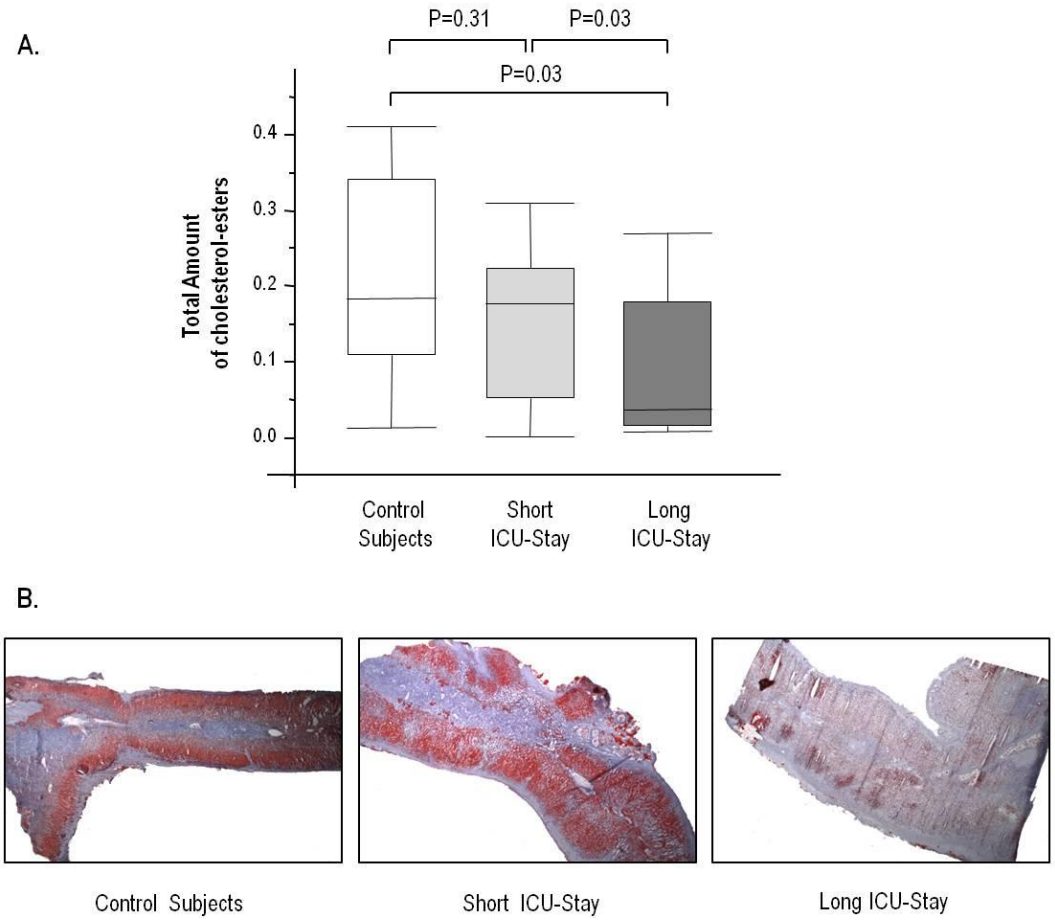

Figure 2: Quantification of the total amount of cholesterol esters in the adrenal glands
*Panel A: The total amount of cholesterol esters was calculated by the product of ORO staining intensity and area. Boxes represent medians and interquartile ranges and whiskers represent 1st quartile-1.5*IQR and 3rd quartile+1.5*IQR. P-values for group comparisons were determined by the Wilcoxon rank-sum test.*
Panel B: The illustrations are representative examples of ORO staining images for each group. Photographs were taken at a 1.25 X magnification.

mRNA expression of ACTH-regulated proteins of the steroidogenic pathway

To assess any confounding effect of post-mortem loss of mRNA quality, the degree of mRNA degradation was investigated. For this purpose, GAPDH mRNA expression was measured with 2 primers directed towards a different exon. There was a tight correlation between the expression using both primers (R^2=0.94; P<0.0001) suggesting that RNA degradation was limited. mRNA expression of NEFL was similar in long ICU-stay patients [0.13 (IQR 0.06-0.77)], in short ICU-stay patients [0.13 (IQR 0.04-1.15)] and in control subjects [0.24 (IQR 0.04-1.70)] (all Ps>0.80), indicating that tissue samples of all study groups contained comparable amounts of adrenal medulla. The amounts of mRNA encoding for MC2R, SCARB1,HMGCR, STAR, and CYP11A1 were at least 58% lower in long ICU-stay patients than in control subjects (all Ps<0.03) (Figure 3). Also compared with short ICU-stay patients, mRNA expression of MC2R, SCARB1, CYP11A1 and STAR was at least 53% lower in long ICU-stay patients (all Ps<0.04). No differences were observed between short ICU-stay patients and control subjects.

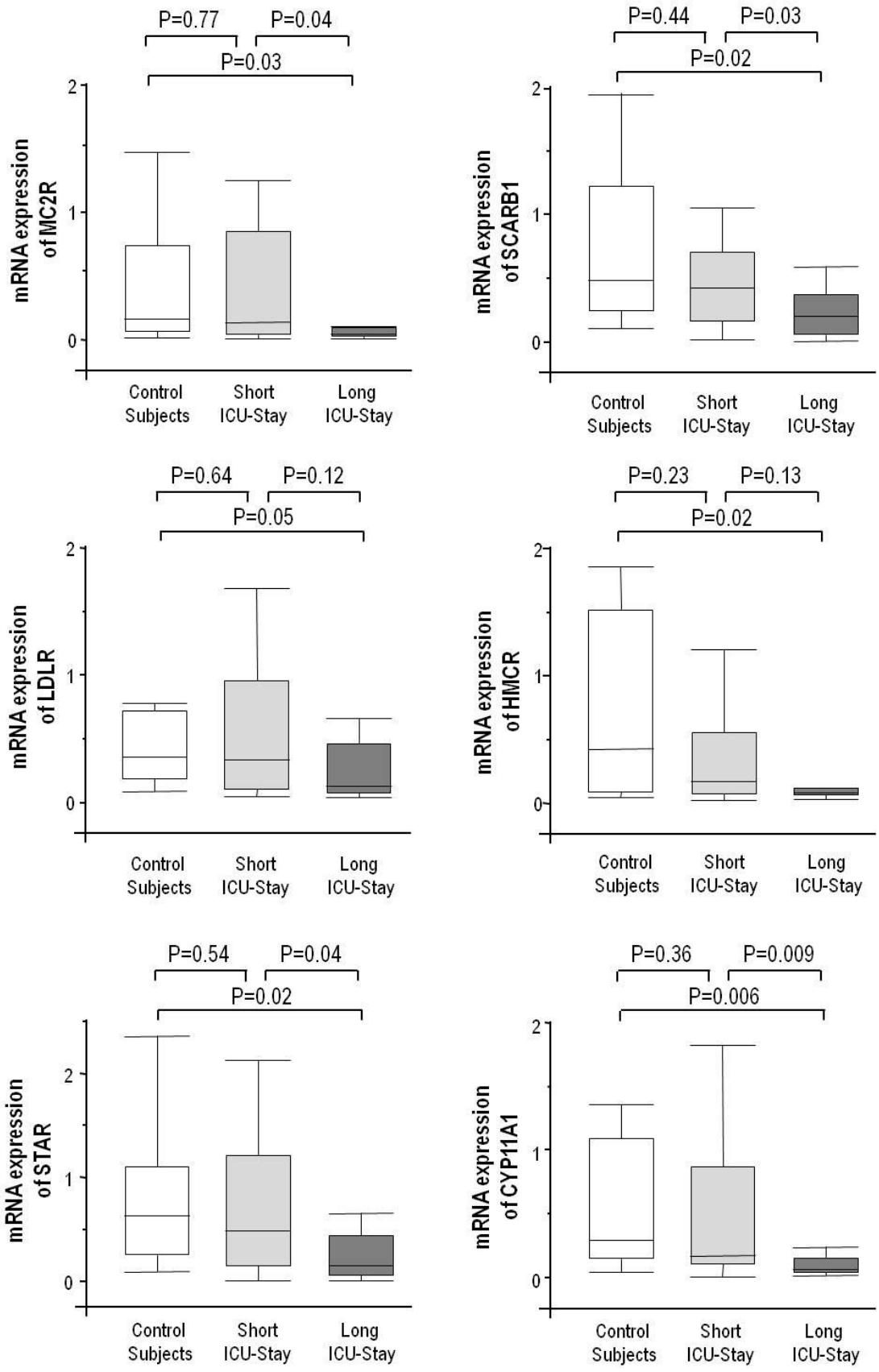

Figure 3: mRNA expression of ACTH-regulated proteins of the steroidogenic pathway

*The mRNA data are expressed, normalized to RNA18S, as a fold difference from the mean of the controls. Boxes represent medians and interquartile ranges and whiskers represent 1st quartile-1.5*IQR and 3rd quartile+1.5*IQR. P-values for group comparisons were determined by the Wilcoxon rank-sum test.*

5.5 DISCUSSION

The results of this human postmortem study suggest that important alterations occur in the adrenal gland during the course of critical illness. In long ICU-stay patients, but *not* in the short stayers, severe cholesterol-ester depletion and substantially reduced mRNA expression of ACTH regulated key-enzymes for steroidogenesis were observed. These observations suggest that with extended duration of critical illness, a significant loss of ACTH signaling in the adrenal cortex may have important functional consequences.

The loss of adrenocortical zonational structure already in the acute phase of critical illness confirmed the results of previous studies performed in small groups of septic patients.[16,17] Although postmortem changes could have played a role, these should then apply to both acute out-of-hospital deaths and to patients who died in the ICU, which was not the case in our study. Furthermore, severe abnormalities were more present in the long ICU-stay patients only. Another interesting observation was that the adrenal glands of long ICU-stay patients tended to weigh less, not more, than those of short ICU-stay patients, while the adrenal glands contained somewhat more water and less protein. This is not what one would expect when the adrenal gland would receive prolonged ACTH stimulation in sustained critical illness, which would cause adrenal hypertrophy and hyperplasia.[18] A prospective study by Jung et al. [19] indeed evaluated *in vivo* adrenal gland volume with computer tomography measurements in septic and non-septic critically ill patients and found increased adrenal gland volume. However, increased blood flow or edema could not be excluded. In post-mortem samples, swollen adrenal glands due to increased blood flow are no longer present which could already explain the differences in observations. Moreover, we only evaluate the sickest patients (those who did not survive); which were the ones with the lowest increase in adrenal gland volume in the study by Jung et al. Polito et al. [17] also evaluated post-mortem macroscopic changes in septic critically ill patients and also observed increased adrenal weight. However, they only evaluated 7 patients, which weakens the generalizability of these conclusions. Hence, as smaller adrenal glands and loss of zonational structure are also observed in POMC-deficient mice,[3] ACTH deprivation with time in ICU could have played a role. Low plasma ACTH concentrations have been repeatedly reported during critical illness[1,20,21] and we recently showed that nocturnal ACTH secretion rate was suppressed in ICU patients.[22] Such ACTH suppression could in part be due to feedback inhibition exerted by elevated cortisol levels brought about by reduced cortisol breakdown.[1] This situation is comparable with sustained exogenous administration of hydrocortisone, which is known to suppress ACTH and with time can cause adrenal atrophy.[23]

Severe depletion of cholesterol esters was only observed in the adrenal glands harvested from long ICU-stay patients and not in those from the short stayers. Since normal adrenocortical synthesis and

release of cortisol relies predominantly on the availability of cholesterol stored in the adrenal cortex, the observed lipid depletion may theoretically contribute to the inability of long ICU-stay patients to acutely release enough cortisol in response to a second hit.[13] Also, the gene expression of one of the cholesterol uptake receptors, SCARB1, as well as of HMGCR, the enzyme responsible for *de novo* cholesterol production, was not increased at all and was significantly down-regulated in the adrenal glands of the long ICU-stay patients only. Furthermore, the expression of genes encoding for the ACTH receptor (MC2R) and the steroidogenic proteins (STAR, CYP11A1) showed a comparable expression profile, with reduced expression levels in long ICU-stay patients only. Given that ACTH stimulates the expression of these genes, sustained ACTH deprivation could explain these findings.[4,5,7,9,10] Indeed, the presentation is again highly reminiscent of the alterations observed in POMC-deficient mice.[12]

Our observation that mRNA expression of the different key genes in steroidogenesis was not upregulated in critically ill patients, does not support a role for ACTH or ACTH-independent stimulators of cortisol synthesis such as neuropeptides, endothelin and cytokines,[2] as all would require an activation of the steroidogenic genes.[24] It actually further supports our previous finding that high plasma cortisol in critically ill patients is to a large extent due to reduced cortisol breakdown.[1, 22] Unfortunately, given the nature of the study, blood samples were not available to correlate the adrenocortical pathology and gene expression findings with plasma ACTH and cortisol concentrations. It appears that especially ACTH *pulsatility* is critical for adequate transcriptional activation of steroidogenic genes.[25] We recently showed, by serial blood sampling and deconvolution analysis, that it is specifically the *pulsatile* nocturnal ACTH release that is suppressed during critical illness, with reduced ACTH pulse mass in the presence of unaltered ACTH pulse frequency.[22] Possibly, in the acute phase of critical illness the pulsatile ACTH signal is still sufficient to prevent downregulation of ACTH responsive genes in the adrenal cortex. However, when increased feedback-inhibition exerted by high cortisol levels is maintained into the prolonged phase of critical illness, and as a consequence pulsatile ACTH is suppressed for a longer time, this might reach a critical threshold below which steroidogenic transcription becomes impaired.

The presented study has several limitations and some strengths. First, inevitably for a human study on this topic, the adrenal glands were harvested at the occasion of autopsy, and although this was performed within 24 hours after death, postmortem artifacts cannot be fully excluded. However, as the corpses were kept cooled until harvesting of the adrenal glands, this problem may have been limited, supported by the mRNA quality analysis that revealed minimal mRNA degradation. Furthermore, not all studied genes were downregulated in the prolonged phase of illness, since LDLR, NEFL and RNA18S5 were unaltered, which speaks against postmortem artifacts explaining the between group differences.

Cholesterol depletion could also theoretically be exacerbated by acute consumption of available cholesterol droplets in the agonal phase of illness.[26,27] However, this would again apply to all ICU patients and to the sudden out-of-hospital deaths alike. Also, adrenocortical lipid depletion was previously observed in an animal model of sepsis-induced critical illness, a model that does not suffer from the above mentioned methodological issues that are inevitable in human studies.[28] Second, acute out-of-hospital deaths used as control subjects were matched for BMI and gender but turned out to be younger than the ICU patients. However, research by Hornsby *et al.* revealed no impact of age on the zona fasciculata.[29] Third, the lack of blood samples did not allow assessing the association between the observed changes in the adrenal glands and the circulating levels of ACTH and cortisol. Hence, *in vivo* animal studies are required to further study the influence of duration of critical illness, and hereby the duration of ACTH deprivation, on adrenocortical morphology and function during critical illness. However, despite these limitations, the strength of this study was that no signs of increased steroidogenesis could be found in the adrenal glands of a large and heterogeneous population of patients, being treated for a variety of illnesses in ICU, with or without sepsis. This observation further corroborated our previous findings *in vivo* indicating that other mechanisms than ACTH drive elevated plasma cortisol concentrations during sustained critical illness.[1,22] Also, this is the first study to investigate the impact of critical illness on the adrenal gland at the tissue level in such a large population. Most previous studies only investigated the adrenal gland indirectly, via the cortisol response to ACTH injection,[30] a test that remains highly debated as to what it reveals about adrenal functional reserve.[31,32]

In conclusion, the changes observed in adrenal glands harvested within 24h after death from long-stay and short-stay ICU patients and from sudden out-of-hospital control deaths suggest that extended duration of critical illness profoundly affects adrenocortical structure and function which could be related to ACTH deprivation. Depletion of cholesterol esters and reduced expression of ACTH-regulated genes involved in steroidogenesis may contribute to the risk of adrenal insufficiency in the prolonged phase of critical illness.

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CHAPTER 6

GENERAL DISCUSSION AND PERSPECTIVES

In part adapted from:

- Boonen E, Van den Berghe G. Endocrine Responses to Critical Illness: Novel Insights and Therapeutic Implications. J Clin Endocrinol Metab 2014 (in press)

- Boonen E, Van den Berghe G. Cortisol Metabolism in Critical Illness: Implications for Clinical Care. Curr Opin Endocrinol Diabetes 2014 (in press)

Despite the general agreement that an adequate HPA-axis function is essential for survival from critical illness, understanding of the pathophysiology of adrenal failure during critical illness is currently incomplete. This precludes further optimization of diagnosis and treatment of this condition which is prevalent among ICU patients. This PhD project has attempted to further clarify the alterations within the HPA-axis as they occur during critical illness, to identify underlying mechanisms of the so-called "ACTH-cortisol dissociation", and to unravel the pathophysiology of adrenal insufficiency in ICU patients.

The classical concept of the HPA stress-response postulates that the elevated plasma cortisol concentrations present during critical illness are primarily, if not exclusively, brought about by a several-fold increased adrenocortical production of cortisol, driven by elevated ACTH and CRH release. (Figure 1) However, published data on plasma ACTH concentrations in critically ill patients are scarce. Vermes *et al.* reported an immediate increase followed by a steep fall in plasma ACTH concentrations after 3 days of critical illness.[1] Interestingly, a small study by Polito *et al.* reported reduced ACTH mRNA levels in 9 human pituitary glands harvested postmortem from patients who died after septic shock as compared with patients who died suddenly from other diseases.[2] This reduced ACTH expression occurred in the absence of a compensatory rise in expression of CRH or vasopressin in the hypothalamus.

We observed in a more heterogeneous critically ill patient population that plasma ACTH concentrations are lower than normal already from ICU admission onward and stay below the lower limit of normality throughout the first week of critical illness.(Chapter 3) This apparent inconsistency in published results may be explained by differences in severity of illness and/or by the type of patients that were included/excluded for the study. Moreover, the specificity of the ACTH assays that were used in the different studies may have differed. Indeed, the ACTH concentrations Vermes *et al* observed were extraordinarily high as compared with other values in the literature, the latter more in the range of the ones we observed in our studies.[3,4] Whether the expected initial ACTH rise in response to stress was missed in our studies and had already occurred before ICU admission, for example in the operating room or emergency department, remains unknown and needs to be further explored. Indeed, other studies performed during and immediately after elective surgery have reported that ACTH is increased during and immediately after surgery, while plasma ACTH concentrations were already normal within the first postoperative day.[5,6] Patients in our studies often entered the ICU some time after the onset of the life-threatening illness, so that the initial rise in ACTH may have been missed. Also studies of children suffering from meningococcal sepsis revealed low plasma ACTH levels already within the first 24h of admission,[7,8] which suggest that tracing the initial ACTH rise presumed to be triggered by severe illnesses could be a major challenge.

The observation of low plasma ACTH concentrations in the face of elevated plasma cortisol, referred to as “the ACTH-cortisol dissociation”, was based on single blood sample measurements of ACTH and cortisol. Given that ACTH and cortisol are secreted in discrete pulses, analyzing the dynamics of the secretory patterns of these hormones and the interaction between ACTH and cortisol secretion must involve time series analyses. We therefore performed a second study (Chapter 4) in which we analyzed the dynamics of ACTH and cortisol secretion via nocturnal time series of repeated (every 10 minutes) blood samples taken from critically ill patients and from healthy controls. By deconvolving these concentration time series into secretion profiles, taking into account the plasma clearance, we revealed decreased ACTH *and* cortisol secretion rates in ICU patients as compared with the healthy controls, which was explained by a decrease in the size of the pulses but not by a change in the number of pulses. Surprisingly the dose-response between a given ACTH concentration and cortisol secretory response was preserved. These findings suggested that the term ‘ACTH-cortisol dissociation’ may not be entirely correct. Indeed, the cortisol secretion was still “connected” to the amount of circulating ACTH, but *both* were suppressed, not increased, in the presence of high plasma cortisol concentrations. The observed low circulating ACTH levels in combination with reduced ACTH and cortisol secretion could be explained by a general suppressor of the HPA axis such as NO or orexin.[2,9] Another possible explanation could be that the circulating high cortisol levels suppress ACTH release centrally by negative feedback which inhibits cortisol production.

Moreover, our data also invalidate the possibility of an increased ACTH sensitivity as an explanation for low ACTH and high cortisol levels, as the preserved dose-response indicated that a given ACTH concentration still drives cortisol secretion during critical illness but does not lead to an increased cortisol response. We also observed an increase in markers of irregularity and asynchrony (ApEn and crossApEn) in the secretory patterns, which further suggested that alternative non-ACTH dependent regulators of cortisol production are involved in the ‘dissociation’.

Pro-inflammatory cytokines are known to stimulate adrenocortical cortisol production and we found both IL6 and TNFα concentrations to correlate positively with the morning cortisol production as determined with the stable isotope tracer (Chapter 3). Remarkably, morning cortisol production rate was only elevated in patients suffering from the ‘systemic inflammatory response syndrome’ (SIRS), while cortisol production in other critically ill patients was indistinguishable from healthy controls, in the presence of elevated plasma cortisol. However, in the study of the overnight cortisol secretion rate, as determined by deconvolution analysis, the plasma cytokine concentrations correlated *inversely* with cortisol secretion (Chapter 4). Hence, the data do not really support a major role for circulating cytokines as key regulators of plasma cortisol. Other potential stimulators of cortisol production such as neuropeptides or

splanchnic innervation were not further investigated. Given the fact that cortisol production was only moderately increased in the morning (Chapter 3), and even decreased during the night (Chapter 4), one could question whether such alternative drivers of cortisol production play an important role during critical illness. This is further supported by the lack of correlation between plasma total and/or free cortisol concentrations and cortisol production rate during critical illness.

Indeed the cortisol production rate during the day, documented with isotopes, was not even a doubling of that of healthy subjects, while the nocturnal cortisol secretion documented was only half that of healthy controls. All together, 24h cortisol production rates during critical illness may not be, or at best only moderately, increased. Ideally, 24h profiles should be constructed in order to quantify the 24h differences in cortisol secretion rates between patients and controls. However, such 24h studies represent a major challenge in the ICU without disturbing daytime diagnostic and therapeutic activities. What can be concluded from our data is that the classical assumption that cortisol production rate during critical illness is 6-fold higher than normal, is probably a large overestimation. As a consequence, the therapeutic doses of 200 mg, currently used for substituting a presumed failing adrenal gland during critical illness, may be too high. This conclusion is supported by the observation that several-fold higher plasma cortisol concentrations were obtained with the treatment of 200 mg hydrocortisone per day of patients who were presumed to suffer from relative adrenal failure, as compared with the endogenous cortisol plasma concentrations in patients who have an adequate HPA-axis response.[10]

As an alternative for increased cortisol production, hypercortisolemia could also be evoked by reduced plasma cortisol clearance. We indeed observed cortisol clearance to be reduced to less than half, as determined with a low dose tracer infusion as well as quantified via the clearance of a 100 mg bolus of exogenous hydrocortisone. Reduced cortisol breakdown was explained by reduced expression and activity of the cortisol metabolizing enzymes in liver and kidney, as suggested by urinary steroid ratios, tracer kinetics and assessment of liver-biopsy samples. (Chapter 3) These results were obtained via 4 different studies, where each of these studies investigated another aspect, making these studies complementary and adding to the robustness of the data. Furthermore, we analyzed possible confounding regulators. Theoretically, as shown in literature,[11,12] CBG levels influence both half-life and clearance of cortisol. We observed in the studied patients decreased CBG levels during critical illness. Low CBG levels would shorten the half-life of cortisol, and increase the plasma clearance and distribution volume of cortisol. Our findings are therefore clearly not explained by alterations in CBG, as the opposite would be expected, which further adds to the robustness of our observations. Decreased plasma clearance of cortisol could also be explained by reduced renal or hepatic function or by

hypoperfusion. However none of the representative clinical markers of organ function and/or perfusion were associated with cortisol plasma clearance. Drugs that interfere with HPA-axis function were all excluded except for anticoagulants and opioids, which are often required during critical illness. We could confidently eliminate potential confounding by such treatments, since there were no differences between patients who were treated with these drugs and those who were not treated.

Importantly, we observed reduced cortisol clearance in all tested critically ill patients, irrespective of type and severity of illness, inflammation status and irrespective of ICU stay and prognosis, which further emphasized the potential key role for this mechanism to increase plasma cortisol. Such adaptation via reducing cortisol breakdown in response to sustained stress and low energy availability is not only observed in critical illness, but has also been described in patients with anorexia nervosa, posttraumatic stress disorders and depression, suggesting that it could be a fundamental part of the stress response.[13-15]

The concept of reduced cortisol metabolism as a vital part of the stress response to critical illness could be interpreted as an attempt of the human body to save energy when energy supply is low. The concept of optimizing energy resources is further supported by low plasma CBG levels in critical illness, causing increased levels of free cortisol, the biologically active form. Importantly, elevating cortisol via the suppression of metabolizing enzymes predominantly in liver and kidney, where cortisol effects are needed for an optimal fight or flight response, could limit the exposure of immune cells and vulnerable target tissues such as skeletal muscle or brain to deleterious side effects of excessive cortisol. Regulating effects of cortisol locally also seems to occur at the level of glucocorticoid receptor (GR) expression. Previous work indeed showed suppressed GR expression in white blood cells of critically ill children, which could be a way to allow the innate immune response to effectively protect the host against infections in the presence of elevated circulating cortisol levels.[16,17] Increasing evidence from animal studies shows that the regulation of the GR in other tissues is also important during critical illness.[18,19]

It remains unclear from our studies, however, what is driving the downregulation of these cortisol metabolizing enzymes in liver and kidney during critical illness. Bile acids, both conjugated and unconjugated, are potent inhibitors of the cortisol metabolizing enzymes, via competitive inhibition at low-physiological concentrations and by suppression of gene and protein expression at elevated concentrations.[20-22] Circulating levels of predominantly conjugated bile acids recently were shown to be substantially increased during critical illness.[23] These elevated bile acid levels appeared to be brought about by ongoing bile acid synthesis, despite suppression of the nuclear receptors that function as

sensors. The bile acids appeared subsequently conjugated within the hepatocyte and exported back towards the blood rather than to the bile. This phenomenon may be adaptive and beneficial as it could reduce energy expenditure by not exporting bile acids against a steep concentration gradient into the bile.[23] Furthermore, bile acids also have an immunomodulatory effect, as shown in septic mice, possibly mediated by their effect on hepatic GR expression.[19] Increasing evidence further emphasizes the beneficial effect of high circulating bile acid levels, since they prevent insulin resistance, promote energy expenditure via increased deiodinase 2 levels in brown adipose tissue[24] and may protect the liver and kidney from ischemia.[25-27] As a strikingly tight inverse correlation was observed between the elevated plasma concentrations of bile acids in patients and the gene and protein expression levels of the A-ring reductases in liver, a role for bile acids in regulating cortisol metabolism during critical illness may likely be an additional beneficial effect of bile acids.[23, 28]

The link between bile acids and the HPA-axis was already suggested from different clinical observations and animal studies. Not only are bile acids and cortisol both produced from cholesterol, they also have 5β-reductase in common which regulates both bile acid synthesis and cortisol metabolism.
Bile acids are suggested to regulate the HPA-axis at every level. Animal experiments showed that cholestasis, besides inhibiting the different cortisol metabolizing enzymes,[21] also reduces both CRH and ACTH expression.[29] Furthermore, the farnesoid X receptor (FXR), a nuclear receptor that is activated by bile acids and downregulated during critical illness,[23] is expressed in the adrenal glands and positively regulates SCARB1, 3β-HSD and CYP11B1 expression, which are important for steroidogenesis.[29-31] This may suggest that besides influencing cortisol metabolism, bile acid homeostasis could also explain our other observations of both decreased ACTH levels and decreased cortisol production. The clinical observations of ACTH-cortisol dissociations in patients with cholestasis [32] as well as the high incidence of adrenal failure in patients with liver diseases [33] further emphasize this potential link.

However, an inverse regulation by glucocorticoids on bile acid homeostasis was also described, since patients with Addison disease have increased circulating levels of bile acids and POMC-deficiency coincides with cholestasis.[34] Moreover, mice studies with deficient hepatic GR expression showed both increased bile acid levels, as well as reduced FXR expression and reduced basolateral uptake of circulating bile acids,[34] which are all findings observed in critically ill patients.[23] Intervention studies are therefore indispensable to further explore this cause-or-consequence debate.

In the last chapter of this thesis, we explored the longer term consequences of the ACTH-cortisol dissociation on the adrenal gland of critically ill human patients. Maintaining plasma cortisol concentrations high by reducing the breakdown of cortisol would cause feedback inhibition on both

pituitary ACTH and hypothalamic CRH release. It has been shown previously that this can predominantly affect pulsatility via reducing the mass per ACTH pulse, which is exactly what we observed in critical illness. (Chapter 4) Reduced pulsatile ACTH secretion was associated with suppressed pulsatile cortisol secretion. There is increasing evidence that emphasizes the importance of hormonal pulsatility in maintaining normal cellular function in both the adrenal gland and the target tissues of cortisol, by preventing desensitization of transcriptional responses. Whether the observed reduced pulsatility in cortisol secretion during critical illness still suffices to prevent such desensitization in target tissues remains unclear. One could speculate that it may indeed contribute to tissue specific cortisol resistance and, by inference, to 'relative' adrenal insufficiency during critical illness.

Whereas reduced cortisol metabolism can be interpreted as an 'economic' way to maintain cortisol levels, the coinciding low plasma ACTH concentrations could negatively affect adrenal structure and function. Therefore, in the last chapter, we investigated post-mortem adrenal glands harvested from patients who died after a short or a long ICU stay, as compared with those harvested from sudden out-of-hospital deaths. The study showed a clear depletion of adrenocortical cholesterol esters and reduced expression of steroidogenic genes (STAR, CYP11A1, MC2R, SCARB1, LDLR, HMGCR) in prolonged critically ill patients, while no changes occurred in acute critically ill patients. These alterations further strengthened our hypothesis that no important activation of the HPA-axis and therefore no increased cortisol production seems to occur during critical illness, since this would upregulate the steroidogenic enzymes. Since cortisol is not stored in the adrenal gland and cortisol secretion relies on immediate cortisol production from cholesterol, these observations instead suggested a reduced cortisol production.

Theoretically, each of the observed changes in the harvested adrenal glands could either be explained by several independent factors or by ACTH alone, as ACTH deprivation can bring about all these alterations. For example, STAR is regulated by several transcription factors and the interactions with proteins such as the peripheral type benzodiazepine receptor (PBR) are known to be essential.[35] STAR is also upregulated by cholesterol depletion. Hence, the low cholesterol levels of the critically ill inferentially should upregulate STAR. Also, STAR knock-out mice are characterized by florid lipid depositions in the adrenal glands.[35,36] However, these adaptations require an increase in ACTH, which is low during critical illness. Furthermore, the cholesterol uptake receptors and HMG-Co A reductase are all negatively regulated by circulating cholesterol. Again, the observed low cholesterol levels during critical illness would therefore rather stimulate cholesterol uptake and synthesis.[37] An adequate supply of oxygen within the mitochondria is known to be essential for CYP11A1 function. Hypoperfusion with ischemia could occur in critical illness. Hypoxia has been previously shown to downregulate both

CYP11A1 and MC2R, however without alterations in STAR expression.[38] Iatrogenic factors such as etomidate are also likely to affect cortisol production. Etomidate is known to inhibit CYP11B, though causes stimulation of STAR and CYP11A1.[39]

All these different regulators cannot explain the full picture of the observed changes in the adrenal gland during critical illness. Since mRNA expression of all the genes correlated tightly with each other (all $P<0.0001$ and all $R^2>0.53$) and mRNA expression of all genes except HMGCR correlated, albeit weakly, with ORO quantification (all $P\leq0.004$ and all $R^2>0.15$), this may suggest that all changes in the adrenal gland are determined by the same regulator during critical illness. ACTH is the only shared regulator of all these alterations; being suggestive that sustained lack of ACTH-effect may indeed explain these findings. Furthermore, predominantly ACTH pulsatility determines transcriptional activation of the steroidogenic genes and we demonstrated that pulsatility is reduced during critical illness. Finally, our results are strikingly reminiscent of the phenotype of POMC-deficient mice.[40,41] Unfortunately, we did not have blood samples to correlate these changes to ACTH plasma concentrations.

The observed changes in the adrenal glands of prolonged critically ill patients, possibly related to ACTH deprivation, may at first sight seem in contrast with our previous finding of a preserved dose-response relationship of ACTH and cortisol. (Chapter4) However, the patients from the study on the dynamics of ACTH and cortisol time series were included early during the course of critical illness (with a median ICU stay at study time of 4 days (IQR 2-5)). In contrast, the observed changes in the study of the adrenal glands were only observed in the prolonged phase, i.e. after a median 16 (IQR 13-21) days in ICU, and not in the adrenal glands harvested from patients with a short ICU stay, i.e. after a median 2 (IQR 1-5) days in ICU. This is consistent with other endocrine changes observed in critical illness, which are likely to be beneficial in the acute phase, but become maladaptive when illness is prolonged.[42,43] Furthermore, Barquist et al. showed a 20-fold increased incidence of adrenal failure in patients with ICU-stay of more than 14 days.[44]

Possibly, other previously suggested regulators such as pro-inflammatory cytokines, impaired blood supply, oxidative stress, cholesterol deficiency or interfering medications could also play a role in the pathophysiology of adrenal failure. For example, since cholesterol uptake and synthesis are downregulated, circulating levels of cholesterol become more important. Indeed it was shown that the circulating HDL-levels are an important prognostic marker of adrenal failure and of adverse outcome of critical illness.[45]

Furthermore, adrenal failure has also been described in the acute phase of sepsis-induced illness. This acute failure can be brought about by other causes of “absolute adrenal failure”, such as iatrogenically decreased adrenal cortisol production by etomidate, adrenal ischemia, adrenal hemorrhage, among

others. As patients often enter the ICU for an acute deterioration of a chronic illness, or were ill already for some time prior to ICU admission, more chronic ACTH deprivation could also play a role in this 'acute' form of adrenal insufficiency.

The novel findings of reduced cortisol breakdown, only moderately increased cortisol production (if at all) and the deleterious impact of sustained ACTH deprivation reshape the current understanding of HPA-axis regulation during critical illness and may have important implications for diagnosis and treatment of adrenal insufficiency.

From large association studies, relative adrenal failure of the critically ill is thought to be identifiable by an insufficient rise (< 9 µg/dl) in plasma cortisol in response to a 250 µg ACTH bolus, irrespective of the baseline plasma cortisol concentration - which is usually much higher than in healthy humans.[46] In such a condition of insufficiently increased cortisol production, a very high plasma ACTH concentration would be expected. However, the recent robust findings that ACTH plasma concentrations are suppressed, that cortisol production is not much elevated, if at all, and that instead reduced cortisol breakdown plays a major role during critical illness, further complicate defining correct diagnostic criteria for adrenal failure. Furthermore, we showed that the cortisol response to ACTH stimulation in critically ill patients correlated positively with both cortisol production rate and cortisol plasma clearance. We also found that patients who reveal a low response to ACTH, to the extent of absolute adrenal failure,[47] were the ones with the most suppressed cortisol breakdown, whereas their cortisol production was still similar to that of healthy subjects. These findings suggest that a low cortisol response to an ACTH injection may simply reflect the degree of negative feedback inhibition exerted by the cortisol that is not broken down. This condition resembles that of patients treated with exogenous glucocorticoids for an extended time, who also reveal a suppressed response to ACTH injection.[48] Whether or not such a low response in the presence of elevated plasma cortisol during critical illness indicates that cortisol availability would be 'insufficient' to cope with the stress of illness remains unclear. Furthermore, it has been shown that the response to an ACTH stimulation test is poorly reproducible in critically ill patients,[49] which further limits the use of a single ACTH stimulation test to diagnose adrenal failure and strengthens the hypothesis that adrenal function during critical illness is dynamic. Therefore, the use of repetitive ACTH stimulation tests could be recommended to both recognize adrenal recovery as well as the evolution to adrenal failure. It has already been suggested that the cortisol response restores after recovery of illness.[50, 51] Furthermore, a retrospective study of de Jong *et al.* showed that patients in whom the cortisol increment to a ACTH stimulation test decreased with time had the worst prognosis.[52] Unfortunately, large enough

and well designed prospective clinical studies of systematic ACTH stimulation tests performed on repeated time points into the prolonged phase of critical illness and recovery are currently lacking.

Reduced cortisol breakdown can be interpreted as a highly energy efficient way to maintain adequate cortisol levels. However, inhibiting cortisol metabolism can only increase plasma cortisol to a limited extent. One could speculate that when a second hit occurs, an additional cortisol production is needed to cope with this extra need of cortisol. If ACTH deprivation was already present for some time so that adrenal function already became compromised, this extra required cortisol may no longer be produced. To detect adrenal failure, repetitive cortisol measurements are needed rather than drawing conclusions from a single cortisol value, taking into account the previously described limitations of total cortisol levels as a reflection of free cortisol and the problems with cortisol assays. Repetitive cortisol measurements in time showed a decrease in serum cortisol levels in the prolonged phase of illness.[53] In this study 25% of the patients who had initial normal baseline cortisol levels evolved to low cortisol levels within a mean time of 8 days, which is consistent with our observation that >7days of ICU stay causes signs of insufficient adrenal cortisol production. It further remains to be studied how ACTH concentrations evolve during the course of critical illness. Whenever a second hit would imply additional cortisol production, high ACTH levels would then be released in an attempt to stimulate adrenal cortisol production and release. However, persistent feedback of high cortisol could also have affected pituitary and hypothalamic function and could cause low ACTH levels. Indeed, long-term administration of exogenous glucocorticoids is known to cause tertiary adrenal insufficiency by prolonged suppression of hypothalamic secretion of CRH,[54] which is comparable with the continuously high cortisol levels during critical illness. Moreover, the observed alterations in adrenal glands from patients, who did not recover, but died after a prolonged phase of critical illness, are highly suggestive of being related to ACTH deprivation.(Chapter 5) On the other hand, by analogy with the evolution of TSH levels in critically ill patients,[43] one could expect that ACTH levels restore when recovery of illness sets in. As such, the changes in circulating ACTH over time could be highly informative to understand illness evolution. Therefore, the combination of repetitive basal (unstimulated) ACTH and cortisol levels would be more informative to assess the function of the HPA-axis during critical illness.

Our new insights also may have important implications for treatment strategies. The proposed dose of 200 mg of hydrocortisone per day, referred to as “low dose” in the literature, is in fact approximately 10 times higher than the normal amount of daily cortisol production in healthy humans [55-58] and between 3- to 6-fold higher than the production which we observed in critically ill patients. Furthermore, the plasma half-life of a bolus of 100 mg hydrocortisone was documented to be at least 5-fold longer in

critically ill patients than in healthy control subjects, and thus hydrocortisone doses of 200 mg per day will likely result in accumulation of cortisol during critical illness. By regulating cortisol breakdown, tissue-specific cortisol levels are ensured which is believed to be beneficial by preventing high levels in those tissues that are susceptible to excessive glucocorticoid levels. Treatment with exogenous glucocorticoids overrules this adaptive response by increasing levels in all tissues, thus explaining the potentially deleterious effect of this treatment. Excessive glucocorticoid levels could inferentially aggravate lean tissue wasting, increase the risk of myopathy and prolong intensive care dependency, which could expose the patients to potentially lethal complications.[59,60] This may further explain why the multi-center randomized controlled study that assessed the effect of hydrocortisone treatment could not confirm the benefit that was originally observed in the pioneer trial.[55,58] Moreover, as glucocorticoid sensitivity could vary among individuals[61] and among cell types [17, 62,63] the dosing issue is further complicated. Also single nucleotide polymorphisms in the GR gene, with an enhanced or decreased response to glucocorticoids, have been identified.[64,65] It thus currently remains a challenge to identify specific clinical biomarkers of GR activation to guide optimal glucocorticoid therapy for individual patients and illnesses.

Further research is urgently needed to investigate appropriate substitution therapy for adrenal failure based on current new insights. The stable isotope studies suggested that a dose of approximately 60 mg of hydrocortisone, equivalent to about a doubling of the normal daily cortisol production, may be interesting to further explore when patients at risk can be identified. A tapering down to the lowest effective dose as soon as possible should limit the adverse effects of excessive amounts of glucocorticoids during critical illness. Furthermore, the novel findings do not only influence treatment of patients with adrenal failure but must also be taken into account when treating ICU-patients with steroids for other indications.

The novel observations described in this thesis generate additional questions which need to be further explored in the future. The evolution of circulating ACTH and cortisol levels during the course of critical illness until recovery or death will give more insight into the evolution of the HPA-axis regulation during critical illness. Ideally this study should be combined with repetitive ACTH-stimulation tests and 24 hour urine collections to evaluate adrenal function and cortisol metabolism during the course of critical illness. Next, well designed clinical trials are needed to define the appropriate treatment strategy of patients with adrenal failure during critical illness. Quantifying the GR expression in different tissues would be of additional interest to explore local tissue sensitivity and further optimize glucocorticoid treatment during critical illness. The impact of sustained high cortisol concentrations at the level of hypothalamus and

pituitary must also be investigated. CRH-stimulation tests have been used to investigate HPA recovery after treatment of Cushing's disease and could be informative in an ICU setting too. Currently, only a limited amount of studies have been performed in critically ill patients, which yielded conflicting results.[66,67] Moreover, also further in detail investigation of the CRH, vasopressin and ACTH expression in relation to reduced cortisol breakdown would be of additional value. However this would imply the need for brain biopsies. Finally, identifying the cause of reduced cortisol clearance and metabolism is indispensable to further understand pathophysiology of endocrine changes during the course of critical illness and recovery.

Human studies are inevitably limited by ethical restrictions. Furthermore, studying post-mortem human material has important limitations. Animal studies eliminate such limitations of post-mortem analyses and further allow in depth investigation of target tissues. Given the possibilities of studying knock-out mice, investigating the HPA alterations during critical illness in a mouse model would be interesting. Our group recently validated a mouse model of sepsis, induced by cecal ligation and puncture.[68] We already completed a small pilot study in 4 healthy control mice and 9 critically ill mice to assess whether the HPA-axis alterations during critical illness in mice are comparable to those observed in humans. Daily blood samples were taken and all animals were sacrificed at the third day of critical illness. ACTH levels were low in critically ill mice compared to controls, while corticosterone levels were always higher in ill mice, suggesting that the "ACTH-cortisol dissociation" is also present in critically ill mice. Furthermore, in liver of critically ill mice, enzyme expression of 5α reductase was markedly reduced, which is important as 5α reductase predominates for cortisol breakdown in mice.[69,70] Though, the sample size of this pilot study was too small and 3 days of critical illness may not be entirely representative for prolonged ICU stay, which is important given that adrenal alterations only occur in the prolonged phase of illness. Therefore, a new larger study with a longer follow-up period is required.

In conclusion, (Figure1) unlike previously inferred, morning cortisol production in critically ill patients was found to be only moderately increased and was suppressed during the night. In the face of low plasma ACTH concentrations and low nocturnal ACTH secretion, these data suggest that other factors drive hypercortisolism during critical illness, which may suppress ACTH by feed-back inhibition. Markedly reduced plasma clearance of cortisol in critical illness, explained by suppressed expression and activity of the main cortisol-metabolizing enzymes in liver and kidney, contributes to high cortisol levels during critical illness. Whereas this stress response could be interpreted as an acute beneficial adaptation of the body to maintain hypercortisolemia in an energy efficient way, sustained low ACTH levels appear to negatively affect structure and function of the adrenal cortex in the prolonged phase of critical illness. These novel insights reshape the current understanding of the hormonal stress response to critical illness. Furthermore, these insights complicate the diagnosis of adrenal failure during critical illness and may suggest a dose adaptation when treatment with hydrocortisone is considered necessary. High quality clinical studies are required to address these many remaining questions.

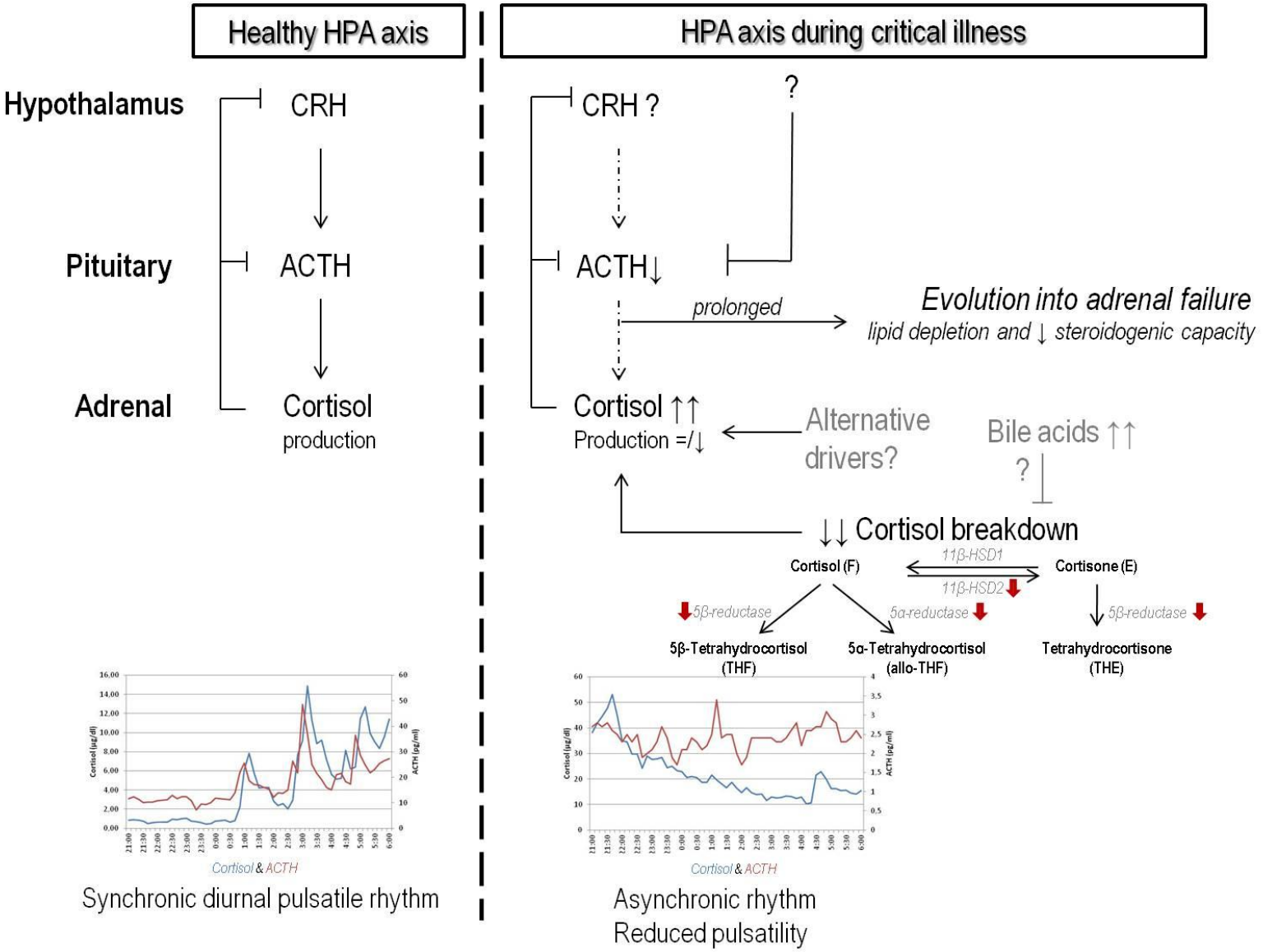

Figure 1 – *Overview of the novel insights and remaining questions in the HPA-axis regulation during and critical illness compared to health*

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SUMMARY

Critical illness is defined as any condition that requires support of failing vital organ functions, without which death would rapidly ensue. As such, it represents an extreme example of physical stress, where "stress" comprises the normal physical response of the human body that arises when confronted with a threat. In healthy individuals, experiencing stress causes an immediate activation of the hypothalamic-pituitary-adrenal axis, which induces secretion of the hypothalamic corticotropin-releasing hormone, followed by the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH causes production of cortisol from cholesterol in the adrenal gland. Cortisol itself exerts feedback inhibition at the pituitary and the hypothalamic level, as such regulating its own release. Under normal conditions, these hormones are secreted in a pulsatile manner according to a fixed daily rhythm. Whenever stress occurs, activation of the hypothalamic-pituitary-adrenal axis causes an additional episodic release of cortisol.

Critical illness is therefore hallmarked by the presence of high circulating cortisol levels, proportionate to the severity of illness. This was traditionally attributed to activation of the hypothalamic-pituitary-adrenal axis and increased ACTH-driven cortisol production. However, ACTH levels were observed to be low, suggesting that non-ACTH dependent regulators of cortisol production are important during critical illness. Cortisol production has even never been quantified during critical illness. Furthermore, it is assumed that activation of the hypothalamic-pituitary-adrenal axis can sometimes be insufficient to cope with severe illness. In the presence of high plasma cortisol, this condition has been labeled "relative adrenal insufficiency". However, the underlying mechanisms remain unknown. Also, it remains controversial whether treating such patients with corticosteroids is required, as interventional studies provided conflicting results.

The main objective of this doctoral thesis was to explore the regulation of cortisol secretion and metabolism during critical illness in order to gain more insight in the pathophysiology of adrenal insufficiency. The general hypothesis of this doctoral thesis postulated that during critical illness reduced cortisol breakdown, rather than continuously stimulated cortisol secretion, contributes to maintain the elevated plasma cortisol concentrations. Negative feedback inhibition, exerted by high circulating cortisol levels would then explain low ACTH levels. When ACTH deprivation sustains, adrenal integrity and function could be negatively affected, predominantly in the prolonged phase of critical illness.

In a first part, we investigated in critically ill patients and matched healthy control subjects daily ACTH and cortisol levels in plasma during the first week of illness; plasma cortisol clearance and production

during infusion of cortisol isotopes as tracers; plasma clearance of 100 mg of synthetic cortisol and the urinary cortisol metabolites and enzyme expression at tissue level to assess the major cortisol-metabolizing enzymes. This showed that while circulating cortisol levels were consistently higher in patients than in controls, ACTH levels were decreased during critical illness. Cortisol production was less than doubled in patients. Furthermore, critically ill patients showed a more than 50% reduction in cortisol clearance during tracer infusion and after the administration of exogenous cortisol. All these factors accounted for the 4-fold increased plasma cortisol levels in patients, as compared with controls. Reduced cortisol metabolism was explained by reduced inactivation of cortisol in the liver and kidney of patients.

In the first study, only single sample ACTH and cortisol concentrations were reported. Since both ACTH and cortisol are secreted in pulses, this precludes analysis of their secretory dynamics and their relation during critical illness. In a second study, we therefore analyzed the dynamics of ACTH and cortisol secretion in critically ill patients and matched healthy control subjects, via nocturnal time series of repeated blood samples. We documented that hypercortisolemia during critical illness coincided with suppressed pulsatile ACTH and cortisol secretion, whereas the cortisol secretory response to a given plasma ACTH concentration was unaltered. These findings speak against the classical dogma of an activated hypothalamic-pituitary-adrenal axis in critical illness and instead suggest feedback-inhibition on ACTH exerted by circulating cortisol.

Considering the important function of ACTH in ensuring adrenal structure and function, the observed low ACTH levels could negatively affect the adrenal glands, possibly predominantly in patients who stayed in the ICU for a prolonged time. In the third part of this thesis, we therefore investigated post-mortem adrenal glands from patients who died in the intensive care unit compared to adrenal glands from sudden out-of-hospital deaths as controls. Adrenal glands from patients who stayed long in the intensive care unit were clearly more distorted in structure. Furthermore, the amount of stored cholesterol droplets was reduced in the adrenal glands from these patients. We also showed that the genes responsible for cholesterol uptake and synthesis as well as crucial genes for the production of cortisol were downregulated only in prolonged critically ill patients. Since cortisol cannot be stored in the adrenal gland and its production is dependent on rapid synthesis from cholesterol, these alterations limit cortisol production. Given that all the observed alterations are regulated by ACTH, one could speculate that the observed low ACTH levels might play a role and, when ACTH deprivation sustains, may help to explain the increased incidence of adrenal failure in the prolonged phase of critical illness.

In conclusion, reduced cortisol metabolism contributes to high cortisol levels during critical illness. Consequently, these high cortisol levels could suppress pulsatile ACTH secretion by negative feedback inhibition, causing acutely a reduced pulsatile cortisol secretion. Maintaining cortisol by not breaking it down seems a highly energy efficient way. However, the concurrent low ACTH levels could explain the observed disruption in structure and function of the adrenal gland in the prolonged phase of critical illness. These novel insights help to understand adrenal failure during critical illness and may have important implications for its diagnosis and treatment.

SAMENVATTING

Kritieke ziekte wordt gedefinieerd als een toestand waarbij de functie van vitale organen moet ondersteund worden om een snel volgende dood te voorkomen. Het lichaam ervaart deze toestand dan ook als een extreme stress situatie. Bij gezonde personen reageert het lichaam op stress door een onmiddellijke activatie van de hypothalame-hypofysaire-bijnier-as, waardoor corticotropine releasing hormoon uit de hypothalamus wordt vrijgesteld. Dit zorgt voor de secretie van het adrenocorticotroop hormoon (ACTH) uit de hypofyse. ACTH stimuleert vervolgens de productie van cortisol in de bijnier. Door negatieve feedback op het corticotropine releasing hormoon en ACTH regelt cortisol zijn eigen vrijzetting. In normale omstandigheden worden deze hormonen vrijgezet in pulsen met een vast dagelijks ritme. In stress situaties zorgt een extra activatie van de hypothalame-hypofysaire-bijnier-as voor een bijkomende vrijzetting van cortisol.

Kritieke ziekte wordt dan ook gekenmerkt door hoge cortisol spiegels, evenredig met de ernst van ziekte. Traditioneel werd dit toegeschreven aan een activatie van de hypothalame-hypofysaire-bijnier-as en een gestegen cortisol productie. ACTH bloedwaarden zijn echter verlaagd in kritieke ziekte, wat lijkt te wijzen op een ACTH onafhankelijke cortisol productie. De cortisol productie werd echter nooit eerder gemeten in kritieke ziekte. Bovendien lijkt de activatie van de hypothalame-hypofysaire-bijnier-as soms tekort schieten tijdens kritieke ziekte, en dit terwijl de cortisol waarden nog steeds hoger liggen dan bij gezonde personen. Deze toestand wordt “relatief bijnierfalen” genoemd. De onderliggende mechanismen van dit falen zijn echter tot op heden onbekend. Aangezien interventionele studies tegengestelde resultaten gaven, is het bovendien onvoldoende geweten hoe men deze patiënten moet behandelen.

Het algemene doel van deze doctoraatsthesis is de regulatie van cortisol secretie en metabolisme tijdens kritieke ziekte te onderzoeken om zo een beter inzicht te krijgen in de pathofysiologie van bijnier-falen. Als algemene hypothese stelden we dat een gedaalde cortisol afbraak, eerder dan een gestimuleerde cortisol productie, de verhoogde cortisol niveaus tijdens kritieke ziekte verklaart. Deze verhoogde cortisol waarden zouden vervolgens een negatieve feedback op ACTH uitoefenen, wat, door een langdurig ACTH tekort, de structuur en functie van de bijnier kan beïnvloeden, vermoedelijk in de verlengde fase van kritieke ziekte.

In het eerste deel van dit doctoraatsproject, onderzochten we bij kritiek zieke patiënten en gezonde vrijwilligers naast de dagelijkse ACTH en cortisol waarden in het plasma tijdens de eerste week van kritieke ziekte, ook de klaring en productie van cortisol aan de hand van een cortisol tracer, de klaring

van 100 mg synthetisch cortisol en de cortisol metabolieten in de urine alsook de enzym expressie in weefsels om zo de belangrijkste cortisol metaboliserende enzymen te onderzoeken. Hieruit bleek dat patiënten steeds verhoogde cortisol waarden hadden ondanks dat ACTH verlaagd was. De cortisol productie was bijna verdubbeld bij patiënten. Bovendien, was de klaring van cortisol uit het bloed meer dan gehalveerd zowel wanneer bepaald na tracer-infusie als na een synthetische cortisol dosis. Samen bepalen ze de viervoudige stijging in plasma cortisol waarden bij patiënten. Een gedaalde afbraak van cortisol in de lever en nieren van patiënten verklaarde het gedaalde cortisol metabolisme.

In de eerste studie werden enkel eenmalige waarden van ACTH en cortisol gerapporteerd. Aangezien zowel ACTH als cortisol in pulsen worden gesecreteerd, verhindert dit de analyse van de secretie dynamiek en hun onderlinge relatie. In een tweede studie analyseerden we daarom de dynamiek van ACTH en cortisol secretie bij zowel kritiek zieke patiënten als gezonde vrijwilligers aan de hand van een serie bloedstalen. We toonden aan dat de hoge cortisol waarden tijdens kritieke ziekte samengaan met een onderdrukte pulsatiele secretie van ACTH en cortisol, terwijl het antwoord in cortisol secretie op een ACTH stimulus onveranderd bleef. Deze observaties staan in sterk contrast met de klassieke veronderstelling dat de hypothalame-hypofysaire-bijnier-as geactiveerd is in kritieke ziekte en suggereren dat de verhoogde circulerende cortisol waarden een negatieve feedback uitoefenen met de verlaagde ACTH secretie tot gevolg.

Gezien de belangrijke functie van ACTH voor de bijnier structuur en functie zouden lage ACTH waarden de bijnieren negatief kunnen beïnvloeden en dit dan vooral bij die langdurig kritiek zieke patiënten. Daarom onderzochten we in een derde deel van deze thesis, post-mortem bijnieren van patiënten die overleden waren op de intensieve zorgenafdeling en vergeleken deze met bijnieren van mensen die onverwacht overleden buiten het ziekenhuis. De bijnieren van langdurig kritiek zieke patiënten hadden duidelijk een meer verstoorde structuur. Verder was het aantal opgeslagen cholesterol druppels in de bijnieren van deze patiënten verlaagd. We konden ook aantonen dat zowel de genen die verantwoordelijk zijn voor de cholesterol opname en synthese als de genen belangrijk voor cortisol productie onderdrukt zijn bij deze patiënten. Aangezien cortisol niet opgeslagen zit in de bijnier en de productie dus afhankelijk is van de snelle omzetting van cholesterol, zorgen deze veranderingen voor een beperking in de cortisol productie. Aangezien al deze veranderingen door ACTH geregeld zijn, doet dit vermoeden dat de aangetoonde lage ACTH waarden een rol spelen en zeker, wanneer deze onderdrukking aanhoudt, mee kunnen verklaren waarom bijnierfalen meer voorkomt in de verlengde fase van kritieke ziekte.

Uit deze studies kunnen we besluiten dat een gedaald cortisol metabolisme bijdraagt tot de hoge cortisol waarden tijdens kritieke ziekte. Hieruit volgt dat deze hoge cortisol waarden door negatieve feedback de pulsatiele ACTH secretie onderdrukken, waardoor acuut de pulsatiele cortisol secretie wordt verminderd. Het behouden van cortisol door het niet af te breken lijkt een zeer energie besparende reactie van het menselijk lichaam op kritieke ziekte. De hierdoor veroorzaakte lage ACTH waarden kunnen bovendien de vastgestelde verstoring van de bijnier structuur en functie verklaren bij patiënten die langdurig op intensieve zorgen verblijven. Deze nieuwe zichten dragen daarom in belangrijke mate bij tot het begrijpen van bijnierfalen tijdens kritieke ziekte en hebben mogelijks belangrijke diagnostische en therapeutische implicaties.

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Publications

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- **Boonen E,** Vervenne H, Meersseman Ph, Andrew R, Mortier L, Declercq PE, Vanwijngaerden YM, Spriet I, Wouters PJ, Vander Perre S, Langouche L, Vanhorebeek I, Walker BR, Van den Berghe G. Reduced cortisol metabolism during critical illness. N Engl J Med 2013;368(16):1477-1488
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Book chapters, internationally recognized scientific publisher

- **Boonen E,** Van den Berghe G. Endocrine Responses to Critical Illness: Novel Insights and Therapeutic Implications. Endocrine and Metabolic Emergencies, Chapter 3, Endocrine Press (In press)

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- **Boonen E**, Meersseman Ph, Vervenne H, Meyfroidt G, Veldhuis JD, Van den Berghe G. Reduced ACTH-driven cortisol secretion during critical illness.
  Poster presentation at the 95th Annual meeting of the Endocrine Society, San Francisco, June 2013. (SAT 50)

Meeting abstracts, presented at local conferences and symposia

- **Boonen E,** Vervenne H, Meersseman Ph, Andrew R, L Mortier, Declercq PE, Vanwijngaerden YM, Spriet I, Langouche L, Vanhorebeek I, Walker BR, Van den Berghe G Mechanisms and implications of markedly reduced cortisol metabolism during critical illness
  Oral presentation at the 22nd Meeting of the Belgian Endocrine Society Liege, Belgium, October 20, 2012.

- **Boonen E**, Vervenne H, Meersseman Ph, Andrew R, L Mortier, Declercq PE, Vanwijngaerden YM, Spriet I, Langouche L, Vanhorebeek I, Walker BR, Van den Berghe G. Reduced Cortisol Metabolism as a Driver of ACTH Suppression during Critical Illness.
  Poster presentation IPSEN Perspectives in Endocrinology 2013